

EFFECTS OF C₆₀ FULLERENE — CISPLATIN COMPLEX ON HONEYBEE *Apis mellifera* L.

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The toxicity of C₆₀ fullerene, traditional cytostatic cisplatin and C₆₀ fullerene-cisplatin complex on honeybee *Apis mellifera* L. toxicity estimation test system was assessed. Water-soluble pristine C₆₀ fullerenes were nontoxic for honeybee when consumed with the food in doses equivalent nontoxic and effective ones for mammalian. Cisplatin toxicity for honeybee in the doses exceed the same for mammalian in 2 times was observed as follows: honeybee 56% death occurred after consumption of 60 mg/kg of bee weight. C₆₀ fullerene-cisplatin complex proved to be more toxic for honeybee in comparison with free cisplatin and caused honeybee 50% lethality after consumption of 40 mg/kg bee weight.

Key words: C₆₀ fullerene, cisplatin, honeybee.

Anticancer drug toxicity on normal highly proliferated tissues is the top problem of the modern medicine. The targeted transport of anticancer agents into the cells is the possible solution to this problem. Such transport could provide therapeutic's total dose reduce and thereafter its toxicity decrease, and maintain and even increase its antitumor efficacy. C₆₀ fullerenes are the prospect delivery vehicles to such transport. They are lipophilic and could penetrate plasma membrane by passive diffusion [1, 2] or endocytosis way [3, 4] and enter into the cell. Therefore the investigation of the biological properties of C₆₀ conjugates with common drugs, in particular with anticancer ones [5–9], is warranted.

C₆₀ fullerene is a spherical-like molecule with a diameter ~0,7 nm consists with carbon atoms [10]. C₆₀ fullerene has an unicum chemical construction and therefore has high reactivity, including addiction to nucleophilic addition reactions [11]. So C₆₀ fullerenes and their derivates can scavenge free radicals including reactive oxygen species (ROS) in biological systems and therefore could be an antioxidants [12, 13]. The ability of C₆₀ fullerene derivates to initiate the cell death by necrotic mechanism in dose-dependent manner was also observed [14]. Toxic properties of C₆₀ fullerenes on human dermal fibroblasts, hepatocytes and normal

astrocytes have been shown to depend on C₆₀ fullerene functionalization [15]. No acute *in vitro* and *in vivo* toxicity of nonmodified C₆₀ fullerenes when act in low concentrations close to physiological ones were shown in [16–18].

As the problem of targeted drug delivery is not solved yet, therefore the investigation of the effects of C₆₀ fullerene — anticancer agent complexes on different test systems to clear the mechanisms of action of such complexes on intact highly proliferated cells is necessary.

Insects gut epithelium is the renewable cell population of varying difficulty depending the insect species, but nevertheless is quite dynamically updated system comparable with gut epithelium of the vertebrate [19]. Therefore the study of the compounds affecting the nucleic acid synthesis and cell proliferation on insects is valid. Honeybee *Apis mellifera* L. is widely used to determine the safety of pollutants for environment. Honeybee is a convenient object for such investigations because of large area of one brood activity (up to 20 km²), genetical homogeneity of individuals from one colony, and their high sensitivity to pesticides, heavy metals and other pollutants. Moreover, the honeybee sensitivity to DNA synthesis inhibitors exceed the same for mammalian in 2–200 times [20].

Antitumor agents could impair DNA synthesis and distribution between daughter cells (alkylating, intercalating agents, antimetabolites, topoisomerase and mitotic spindle inhibitors) or affect the cell signal transduction (hormones and antihormones, protein kinases inhibitors) [21]. Notably, that the first are much more toxic to normal cells compared to the last [22]. Cisplatin (cis-Diamminedichloroplatinum(II), CisPt) is one of the most common alkylating agent, widely used for therapy of the solid tumors [21]. CisPt forms coordination bonds between Platinum atom and two guanine residues of DNA (N^7 atoms) or protein SH-groups, called "platinum adducts" and thereby alters DNA. So DNA-DNA and DNA-protein links appear and SH-comprising enzymes are inactivated. At the cell level CisPt alters DNA replication and transcription and thereby cell cycle arrest and apoptosis occur. Therefore morphological injuries are observed predominantly in highly proliferated tissues.

In this work the investigation of C_{60} fullerene, CisPt and C_{60} fullerene-CisPt complex (C_{60} +CisPt) water solutions in honeybees acute oral toxicity test was aimed.

Materials and Methods

Honeybees *Apis mellifera L.* acute oral toxicity test was performed [23]. The young adult worker bees of Ukrainian steppe race from the healthy colony were used. Insects were collected at the day of experiment and randomly allocated to the entomological cages sized $10 \times 10 \times 15$ cm of 10 bees per cage. The bees were held in the dark (except the time needed for treatment and observations) at a temperature of 23 ± 1 °C and the relative humidity 40%. Eppendorf plastic tubes with hermetic plug and 2 mm hole in the bottom were mounted into the cage roofs and used as feeders.

The stable C_{60} fullerene water colloid solution (0,15 mg/ml) was prepared as described [24, 25]. Structure characteristic of prepared solution was performed using atomic force microscopy (AFM, commerce system Solver Pro M; NTMDT, Russian Federation).

CisPt (Cisplatin-TEVA, PHARMACHEMIE BV, Netherlands, mature concentration 0,5 mg/ml) was dissolved in saline down to 0,15 mg/ml.

C_{60} +CisPt complex was prepared by mixing of C_{60} fullerene (0,15 mg/ml) and CisPt (0,15 mg/ml) water solutions in the volume ratio of 1:1. Obtained mixture was processed in an ultrasound disperger for 10 min followed by magnetic mixer processing for 18 h at the room temperature. Our previous model settlements

suggest the stability of C_{60} +CisPt complex in water solution [9].

Water solutions of C_{60} fullerene (0,15 mg/ml), CisPt (0,15 mg/ml) and C_{60} +CisPt (0,075 + 0,075 mg/ml) were mixed with sucrose syrup and distilled H_2O to obtain 33% sucrose, 0,075 and 0,0375 mg/ml C_{60} fullerene, 0,075 and 0,0375 mg/ml CisPt, 0,075 + 0,075 and 0,0375 + 0,0375 mg/ml C_{60} +CisPt in final liquids. Feeders were filled up by 1 ml of tested solutions per day. Pure 33% sucrose syrup was used as a control. Bees were observed after 24, 48 and 72 h from the start of experiment, the feeders were weighted, the amounts of treated diets consumed per group were monitored, and the amounts of tested substances consumed per bee were calculated. Insects were suggested as alive when any motion was observed, otherwise they were suggested as dead [23]. Three replicate test groups, each of ten bees, were dosed with each test concentration and control.

Statistical analysis was carried out using SPSS 17.0 software for Windows.

Results and Discussion

The obtained AFM image (Fig.1) demonstrates that as single C_{60} fullerenes as their aggregates with size 3–200 nm were attended in water solution, which is agreed with [26].

There were no dependence of consumed food from concentration of tested chemicals, so no repellent properties of C_{60} fullerene, CisPt and C_{60} +CisPt solutions were observed.

The maximum amount of consumed

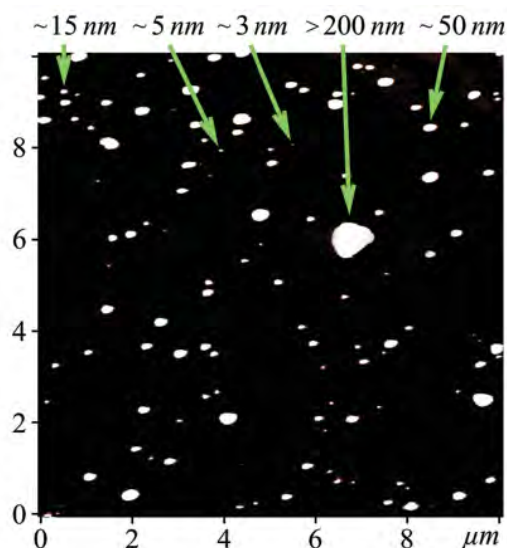


Fig. 1. AFM image (semi-contact mode) of C_{60} fullerene aggregates precipitated on mica from water solution: 0,15 mg/ml (3 months after preparation)

through 72 h C₆₀ fullerene was 7,5 µg per bee, or 75 mg/kg of bees [27]. There were no bees mortality in C₆₀ fullerene group, as in the control one, suggesting the safety of C₆₀ fullerene (0,075 and 0,0375 mg/ml) water solutions for insects and agreeing with [28]. Thus, peroral and intraperitoneal maximum tolerant dose of C₆₀ fullerene for rats was 5 g/kg body weight and excreted at 48–96 h [29].

Bees mortality was observed after consumption of CisPt and C₆₀+CisPt solutions after 72 h of exposure. Bees mortality dependences from consumed dose of CisPt are depicted in Fig. 2.

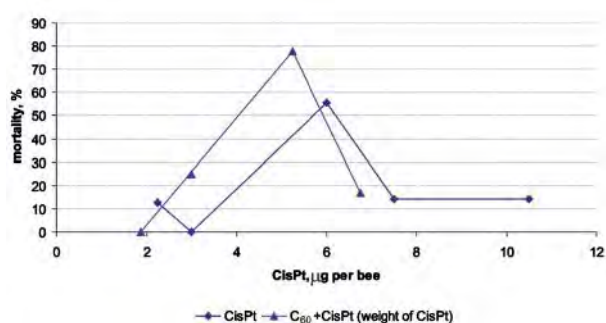


Fig. 2. Honeybee mortality dependence from consumed doses of free CisPt and C₆₀+CisPt complex after 72 h of exposure

As we can see from the Fig. 2, the dose of CisPt caused the maximum bees mortality (56%) is 6 µg per bee or 60 mg/kg of bees, or, if calculated as described at [30], 18,3 mg/m² of body area. For comparison, CisPt LD₅₀ for mice is 39 mg/m² of body area (or 13,5 mg/kg body weight) [21], suggesting the bees sensitivity to CisPt exceed the same of mammalian in 2 times.

Bees mortality dependence from consumed dose of CisPt is domical, which is unexpective (Fig. 2). The possible reason is, on our opinion, the impairment of CisPt transportation into the cell. Three ways of CisPt import into the eucariotic cell are known [31]: passive diffusion, caused by neutrality of CisPt molecule, transportation by organic cation transporter 2 (OCT2), and by Copper influx transporter CTR1 [31]. Notably, that the last

plays the main role in CisPt influx. *In vitro* investigations showed that CisPt bounds irreversibly with Methionine-rich motifs of CTR1 transmembrane domains, so inactivation of CTR1 as well as the drug follows. *In vivo* transportation is possible through continuous flow of Copper cations, capable to catalytically cleave the bounds between Platinum (CisPt) and Sulfur (Methionine residues) atoms [32]. Therefore CisPt transportation occurs with the Copper one simultaneously and, moreover, depends from it. We could suggest that, if CisPt input into the bee organism increases, physiological Copper influx cannot promote the free CisPt transportation into the cell by CTR1 through catalysis of CisPt-Methionine bounds cleavage. Some bounds probably persist, so CTR1 could be inactivated and further CisPt input into the cell could be stopped. Therefore if CisPt input into the organism would increase, the drug influx into the cell would lessen, so the effect on the organism would be nonlinear.

We also observed the shift of bees mortality curve if consumed C₆₀+CisPt complex relative to free CisPt (Fig. 2) to lower doses: maximum bees mortality (almost of 80%) occurs when consumed of 4,6 µg per bee or 46 mg/kg of bees of CisPt in the C₆₀+CisPt complex, whereas only 45% mortality occurs when consumed the same dose of free CisPt. Such shift could be explained by C₆₀ fullerene properties as drug delivery vehicle [33]: probably, CisPt influx into the cell could be increase through its passive diffusion and/or endocytosis in the C₆₀+CisPt complex.

Obtained *in vivo* results should us to conclude the next:

1. Water-soluble pristine C₆₀ fullerenes are nontoxic for honeybee *Apis mellifera L.* when consumed with the food in doses equivalent nontoxic and effective ones for mammalian (0,075 mg/ml of food).

2. Cisplatin toxicity for honeybee *Apis mellifera L.* exceeds the same for mammalian in 2 times: 56% bee mortality occurred after consumption of 60 mg/kg of bees.

3. C₆₀ fullerene-cisplatin complex proved more toxic for honeybee *Apis mellifera L.* in comparison with free cisplatin and caused honeybee 50% mortality after consumption of 40 mg/kg of bees.

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ЕФЕКТИ КОМПЛЕКСУ С₆₀-ФУЛЕРЕНУ ІЗ ЦИСПЛАТИНОМ НА МЕДОНОСНИХ БДЖІЛ *Apis mellifera* L.

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Оцінено токсичність С₆₀-фулерену, традиційного цитостатика цисплатину, та комплексу С₆₀-фулерену із цисплатином на тест-системі оцінювання токсичності — медоносній бджолі *Apis mellifera* L. Показано, що водорозчинні С₆₀-фулерени є нетоксичними для медоносних бджіл за споживання з кормом у кількостях, які відповідають ефективним нетоксичним дозам для ссавців. Встановлено також, що цисплатин є токсичним для медоносної бджоли у кількості, що в 2 рази нижча за аналогічну для ссавців, спричинюючи 56% -ну летальність за умов споживання з кормом у дозі 60 мг/кг маси або 18,3 мг/м² площі поверхні тіла бджіл. Комплекс С₆₀-фулерену із цисплатином виявився більш токсичним для медоносної бджоли порівняно з вільним цисплатином, зумовлюючи 50% -ну летальність за введеної з кормом дози 40 мг/кг маси бджіл.

Ключові слова: С₆₀-фулерен, цисплатин, медоносна бджола.

ЭФФЕКТЫ КОМПЛЕКСА С₆₀-ФУЛЛЕРЕНА С ЦИСПЛАТИНОМ НА МЕДОНОСНЫХ ПЧЕЛ *Apis mellifera* L.

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Оценена токсичность С₆₀-фуллерена, традиционного цитостатика цисплатина, и комплекса С₆₀ фуллерена с цисплатином на тест-системе оценки токсичности — медоносной пчеле *Apis mellifera* L., чувствительность которой к веществам, подавляющим синтез ДНК, превышает чувствительность млекопитающих в 2–200 раз. Показано, что водорастворимые С₆₀-фуллерены нетоксичны для медоносных пчел при потреблении с кормом в количествах, которые соответствуют эффективным нетоксичным дозам для млекопитающих. Установлено также, что цисплатин токсичен для медоносной пчелы в количестве, меньшем за аналогичное для млекопитающих в 2 раза, вызывая 56% -ю летальность при условии потребления с кормом дозы 60 мг/кг массы или 18,3 мг/м² площади поверхности тела пчел. Комплекс С₆₀-фуллерена с цисплатином оказался более токсичным для медоносной пчелы по сравнению со свободным цисплатином, вызывая 50% -ю летальность при введении с кормом дозы 40 мг/кг массы пчел.

Ключевые слова: С₆₀-фуллерен, цисплатин, медоносная пчела.