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# EFFECTS OF $C_{60}$ FULLERENE — CISPLATIN COMPLEX ON HONEYBEE *Apis mellifera* L.

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The toxicity of $C_{60}$ fullerene, traditional cytostatic cisplatin and $C_{60}$ fullerene-cisplatin complex on honeybee *Apis mellifera* L. toxicity estimation test system was assessed. Water-soluble pristine $C_{60}$ 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 fallows: honeybee 56% death occurred after consumption of 60 mg/kg of bee weight. $C_{60}$ 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:*** $C_{60}$ 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. $C_{60}$ 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 $C_{60}$ conjugates with common drugs, in particular with anticancer ones [5–9], is warranted.

$C_{60}$ fullerene is a spherical-like molecule with a diameter ~0,7 nm consists with carbon atoms [10]. $C_{60}$ fullerene has an unicum chemical construction and therefore has high reactivity, including addiction to nucleophilic addition reactions [11]. So $C_{60}$ 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 $C_{60}$ fullerene derivates to initiate the cell death by necrotic mechanism in dose-dependent manner was also observed [14]. Toxic properties of $C_{60}$ fullerenes on human dermal fibroblasts, hepatocytes and normal astrocytes have been shown to depend on $C_{60}$ fullerene functionalization [15]. No acute *in vitro* and *in vivo* toxicity of nonmodified $C_{60}$ 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 $C_{60}$ 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 $km^2$), genetical homogeneity of individuals from one colony, and their high sencitivity to pecticides, heavy metals and other pollutants. Moreover, the honeybee sencitivity 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 agnts, 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 "platimum 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 10×10×15 sm 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 temperatue. 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

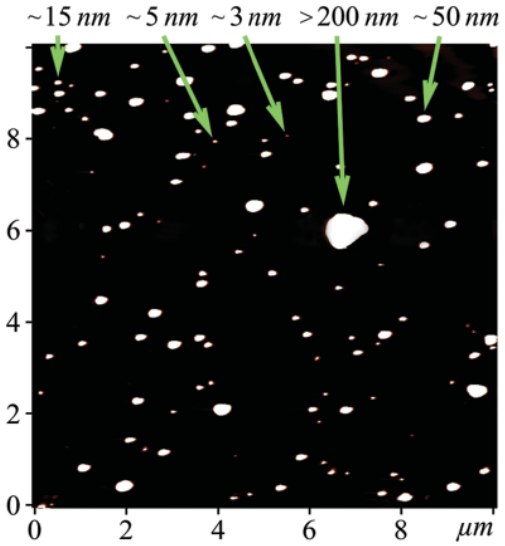


*Fig. 1.* **AFM image (semi-contact mode) of $C_{60}$ fullerene aggregates precipitated on mica from water solution: 0,15 mg/ml**
(3 months after preparation)

through 72 h $C_{60}$ fullerene was 7,5 μg per bee, or 75 mg/kg of bees [27]. There were no bees mortality in $C_{60}$ fullerene group, as in the control one, suggesting the safety of $C_{60}$ fullerene (0,075 and 0,0375 mg/ml) water solutions for insects and agreeing with [28]. Thus, peroral and intraperitoneal maximum tolerant dose of $C_{60}$ 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 $C_{60}$+CisPt solutions after 72 h of exposure. Bees mortality dependences from consumed dose of CisPt are depicted in Fig. 2.

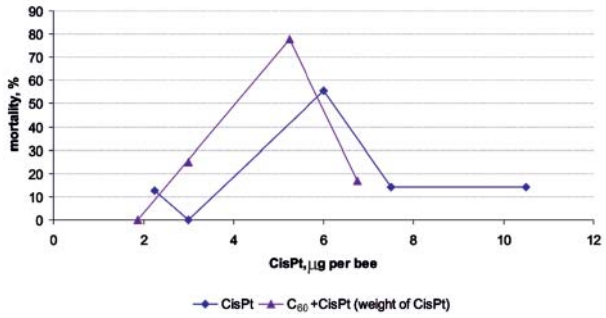


*Fig. 2.* **Honeybee mortality dependence from consumed doses of free CisPt and $C_{60}$+CisPt complex after 72 h of exposure**

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/m$^2$ of body area. For comparison, CisPt $LD_{50}$ for mice is 39 mg/m$^2$ 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 continious flow of Copper cathions, 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 $C_{60}$+CisPt complex relative to free CisPt (Fig. 2) to lower doses: maximum bees mortality (almost of 80%) occurs when comsumed of 4,6 μg per bee or 46 mg/kg of bees of CisPt in the $C_{60}$+CisPt complex, whereas only 45% mortality occurs when comsumed the same dose of free CisPt. Such shift could be explained by $C_{60}$ 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 $C_{60}$+CisPt complex.

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

1. Water-soluble pristine $C_{60}$ 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. $C_{60}$ 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 $C_{60}$-ФУЛЕРЕНУ ІЗ ЦИСПЛАТИНОМ НА МЕДОНОСНИХ БДЖІЛ *Apis mellifera* L.

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Оцінено токсичність $C_{60}$-фулерену, традиційного цитостатика цисплатину, та комплексу $C_{60}$-фулерену із цисплатином на тест-системі оцінювання токсичності — медоносній бджолі *Apis mellifera L.* Показано, що водорозчинні $C_{60}$-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/м$^2$ площі поверхні тіла бджіл. Комплекс $C_{60}$-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:*** $C_{60}$-фулерен, цисплатин, медоносна бджола.

## ЭФФЕКТЫ КОМПЛЕКСА $C_{60}$-ФУЛЛЕРЕНА С ЦИСПЛАТИНОМ НА МЕДОНОСНЫХ ПЧЕЛ *Apis mellifera* L.

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Оценена токсичность $C_{60}$-фуллерена, традиционного цитостатика цисплатина, и комплекса $C_{60}$ 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 $C_{60}$-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/м$^2$ площади поверхности тела пчел. Комплекс $C_{60}$-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:*** $C_{60}$-фуллерен, цисплатин, медоносная пчела.