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# Evaluation of Biological Effects of Graphene Oxide Using *Drosophila*

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Graphene and its derivatives have attracted great interest because of their intriguing physical and chemical properties. An increasing phase of commercial production causes the presence of graphene in the environment and might pose a great threat to a wide range of living organisms, including bacteria, viruses, plants, invertebrates and mammals, including humans. In the present study, the graphene oxide (GO) at low doses was evaluated for its biological effects on larvae and the imago of *Drosophila melanogaster*. Oral administration of GO at concentrations of 0.02 - 1 % has a beneficial effect on the developmental rate and hatching ability of larvae. Long-term administration of a low dose of GO extends *Drosophila* lifespan, improves fecundity rate and significantly enhances resistance to environmental stresses. We also found that GO exposure led to a remarkable decrease in the level of hemolymph glucose, glycogen and triglycerides storage, and, consequently, GO effects on carbohydrate and lipid metabolism in adult *Drosophila*. These findings might provide a useful reference to assess the biological effects of GO, which could play an important role in a variety of graphene-based biomedical applications.

**Keywords:** graphene oxide, *Drosophila*, oxidative stress, metabolism, lifespan.

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## Introduction

Graphene, a single layer of graphite, is a sensational nanocarbon with unusual properties. Graphene is a two-dimensional planar and hexagonal array of carbon atoms [1]. Monocrystalline graphitic films were discovered by Andre Geim and Konstantin Novoselov in 2004 [2] that were awarded the Nobel Prize in Physics in 2010 “for groundbreaking experiments regarding the two-dimensional material graphene”. All graphene properties make it a potential candidate for numerous applications in nanoelectronics and energy technology (it can be used for supercapacitors, batteries, and composites) as sensors, and for biomedical uses like biosensors [3], drug and gene delivery [4], including absorption of enzymes [5], cell and tumor imaging, cancer photothermic therapy, energy storage [3, 6], photocatalysis [7], and fuel cells [8, 9].

Graphene oxide (GO) and reduced graphene oxide (rGO) as valuable graphene derivatives show different chemical and structural properties. While the presence of graphene derivatives becomes more widespread and commonplace across the biomedical sciences, their concentrations in ecosystems are not reported yet. The relatively larger body of work detailing the biological effects of GO on living organisms [10, 11]. However, the impact of graphene on model organisms is very controversial and is a hot topic of discussion. *Drosophila melanogaster* has attracted attention as a model system for evaluating the physiological effects of various chemicals. It was recently shown significant behavioral and developmental disorders in *Oregon-R* flies after GO exposure at a very high concentration (50 - 300 μg/mL) [12]. Another experimental study demonstrated that GO exposure exerted remarkable toxicities in $w^{1118}$ flies [13].

In this context, we evaluated GO at low doses for the physiological and metabolic effects on the fruit fly *Drosophila melanogaster*. Graphene oxide at a concentration of 0.02 - 1 % showed beneficial effects on *Drosophila* physiology. We suggested the involvement of the hormetic effect and assumed that long-term exposure

to a low dose of GO serves as mild stress for the activation of the defense system in flies protecting from subsequent stressful conditions.

# I. Experimental details

***Insects, maintaining and conditions***

*Canton-S* flies *D. melanogaster*, obtained from the Bloomington Stock Center (Indiana University, USA), were grown on the standard yeast-corn medium at 25 °C, with a relative humidity of 60 - 70 % and 12 h day/night cycle. Flies aged two days were separated by sex and kept on the above-mentioned medium for one more day for recovery after $CO_2$ anesthesia. On the next day, flies were placed at standard densities of 200 flies per 1.5 L demographic cages attached with a 25 mL plastic vial filled with the 5 mL of medium [14]. Food contained 5 % sucrose, 5 % dry yeast, 1.2 % agar, 0.18 % nipagin. Media was supplemented by different concentrations of GO: 0.02, 0.04, 0.1, 0.4 and 1 %. On the 15$^{th}$ day of the experiment, flies were used for physiological tests or frozen in liquid nitrogen for biochemical measurements.

***Graphene oxide (GO).*** GO of the highest degree of purity and of specific flakes lateral size was purchased in Grafren AB (Sweden). Highly dispersible in water. GO was synthesized using the modified Hummers approach [15].

***Pupation and pupation height***

Effects of GO on fly development were evaluated by estimating pupation time and pupation height [16]. Flies aged 3 - 7 days were subjected to 3-hour starvation with subsequent 15-hours eggs-laying on the medium composed of 5 % sucrose and 2 % agar. To prevent effects caused by larvae density, laid eggs were washed three times with distilled water, then concentrated in a volume of 1.5 mL and transferred into bottles containing 25 mL of experimental medium (150 - 250 eggs). The number of pupae formed from larvae fed experimental media was counted every 6/6/12 hours (at 9am, 3pm, 9pm) until the end of pupation [17]. The distance from the food surface to each pupa was measured. The pupation height was expressed in millimeters (mm).

***Lifespan.*** Newly eclosed flies were transferred into fresh food and kept for three days for mating. Then flies were separated by sex under light $CO_2$ anesthesia and kept for another day for recovery. About 100 flies of each sex were gently transferred to 1.5 L demographic cages with an attached plastic vial filled with 5 mL control food or experimental food supplemented with different concentrations of GO. Food was changed every second day, and dead flies were removed and recorded. The experiment was run in two replicates [18].

***Fecundity.*** One female and two male flies were placed into glass vials (5 mL) with 1 mL of medium. Food was changed every day and the number of eggs laid by females was counted each day for 5 days. The average amount of eggs was calculated per fly and expressed as eggs/fly/day.

***Metabolites pool.*** Experimental flies were used to measure concentrations of glucose in hemolymph and glycogen levels as well as triglycerides content as described earlier [19, 20].

***Resistance to starvation and oxidative stress.*** Starvation resistance was measured in flies given only 1 % agar as a food source. To study resistance to oxidative stress flies of each experimental cohort were transferred into empty vials for 2 h for starvation. After starvation, flies were transferred into vials containing folded and rammed strips (2.4 × 12 cm) of 4-layer cellulose filter paper soaked with 0.8 mL of 20 mM menadione in 5 % sucrose solution [21]. Fly viability was checked daily at 9am, 3pm and 9pm. Stress resistance was expressed as the percentage of flies that survived over time. Each experiment was run in two biological replicates with 30 flies tested in each replicate.

***Statistical analysis and graphical representation***

Experimental data are presented as mean ± SEM and $p < 0.05$ is considered significantly different. Statistical analysis and graphs were performed using Graphpad Prism 7 (GraphPad Software, Inc.). Dunnett's multiply comparison test has been used to compare the data. Survival curves were compared by a Log Rank test. Maximum lifespan was calculated as the mean of the 10 % of flies that had the longest lifespan.

# II. Results

***Effects of GO on Drosophila during development***

To investigate GO toxicity, we first measured the pupation rate and pupation height. We found that developmental time did not depend on GO supplementation to the nutrient medium at the concentration range of 0.02 - 1 % (Figure 1A).

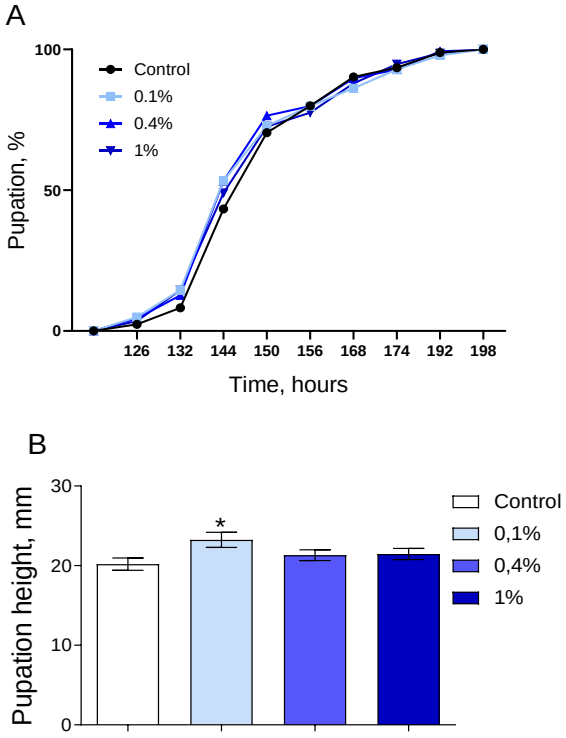


**Fig. 1.** Developmental pattern of *D. melanogaster* reared on media supplemented by GO: (A) developmental rate; (B) pupation height. Graphs show the percentage of larvae that pupated over time. Data are mean ± SEM, n = 6. Group comparisons were performed using Dunnett's test. Asterisk indicates a significant difference between groups with $p < 0.05$.

Pupation height, defined as the distance from the medium surface to each pupa, was slightly increased (by ~15 %) upon feeding with a 0.1 % GO diet (Dunnett's test, $p = 0.02$), whereas pupation height of 0.4 - 1 % GO diet cohorts were unchanged (Figure 1B).

***Effects of GO on lifespan***

We next investigated the effects of long-term exposure to GO on the adult fly lifespan. The lifespan of flies fed by diets supplemented with 0.02 - 1 % of GO was significantly decreased in both sexes (Figure 2 A, B). We observed a longer lifespan in males who consumed media with GO at all experimental concentrations as compared to the control group (Fig. 2A; log-rank test, $p < 0.005$). Similarly, females fed by 0.04 - 1 % GO-containing media displayed a longer lifespan as compared to the control (Fig. 2B; log-rank test, $p < 0.0001$).

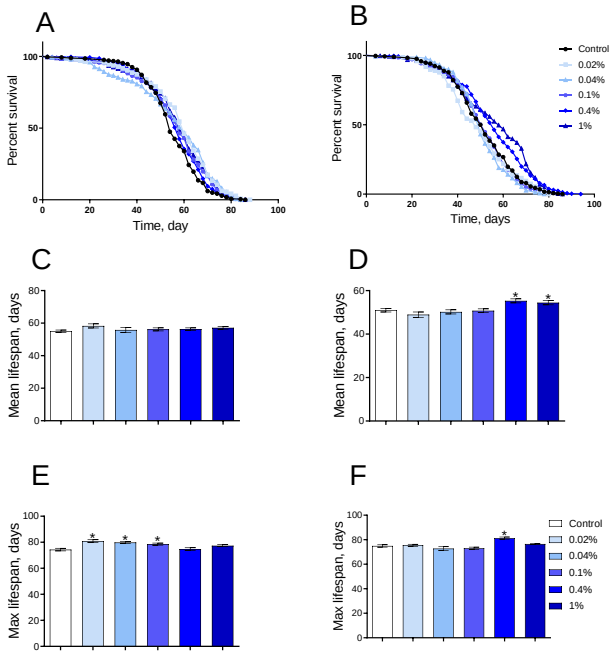


**Fig. 2.** Survival (A – males; B – females), mean lifespan (C – males; D – females) and maximum lifespan (E – males; F – females) of flies exposed to GO. In A and B, each curve represents the proportion of individuals alive as a function of age for about 200 flies. Data are mean ± SEM. Group comparisons were performed using Dunnett's test. Asterisk indicates a significant difference between groups with $p < 0.05$.

The mean lifespan of control female flies was 50.8 ± 0.82 days. Whereas, females fed by 0.4 and 1 % of GO-supplemented diets displayed a mean lifespan of 54.8 ± 0.92 and 54.5 ± 1.01 days, respectively (~ 8 % decrease compared to control, Dunnett's test, $p < 0.05$) (Fig. 2D). We also observed a significantly higher maximum lifespan in male flies which consumed media with GO at the concentrations of 0.02 - 0.1 % (Fig. 2E; Dunnett's test, $p < 0.005$). The maximum lifespan of control females was 74.9 ± 0.95 days, whereas the maximum lifespan of females who consumed the medium with 0.4 % of GO was 81.3 ±0 .88 which is significantly higher compared to the control (Fig. 1F Dunnett's test, $p < 0.0001$).

***Effects of GO on the reproduction***

To further analyze the effects of GO on the physiology of *Drosophila*, we investigated the effects of GO on female fertility. We found that GO induces egg-laying activity in *Drosophila*. Control females laid nearly 4.0 ± 0.45 eggs daily. Females fed by a diet supplemented with 0.04 – 1 % of GO laid approximately 1.5 - 2-fold more eggs as compared to control (Fig. 3; Dunnett's test, $p < 0.05$).

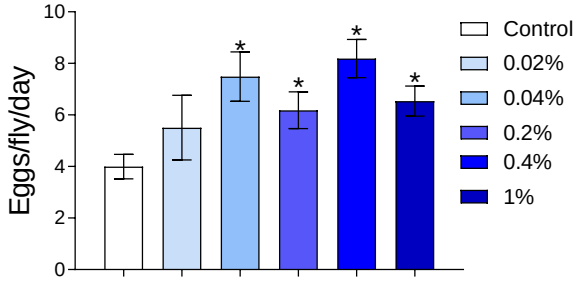


**Fig. 3.** The effects of GO in the diet on the fecundity in *Drosophila*. Results represent the mean ± SEM of 10 replicates per group. Group comparisons were performed using Dunnett's test. Asterisk indicates a significant difference between groups with $p < 0.05$.

***Effects of GO on metabolism***

Glucose, glycogen and TAG are parameters extensively used as measures of carbohydrate and fat metabolism. GO supplementation to the media at the concentrations of 0.04 - 1 % resulted in 30 - 35 % lower levels of glucose in the hemolymph of females (Fig. 4B; Dunnett's test, $p < 0.05$). The hemolymph glucose level in males was not affected by dietary GO (Fig. 4A).

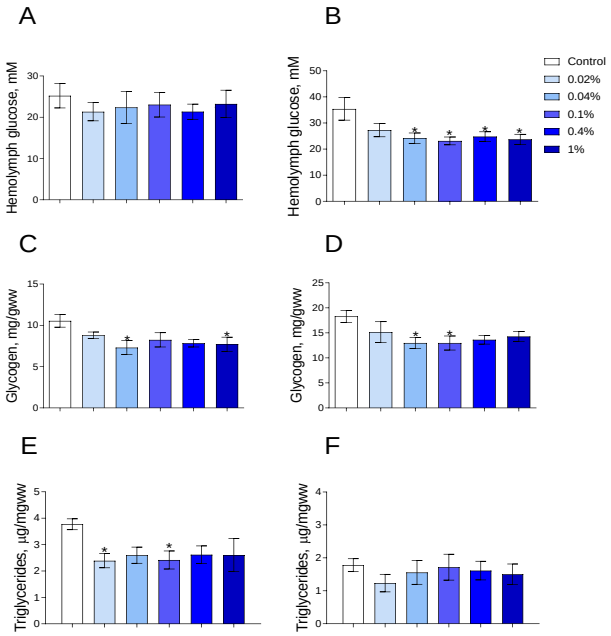


**Fig. 4.** Levels of hemolymph glucose (A – males; B – females), glycogen (C – males; D – females) and triglycerides (E – males; F – females) in adult *Drosophila* exposed to food supplemented by GO. Results represent the mean ± SEM of 4-6 replicates per group. Group comparisons were performed using Dunnett's test. Asterisk indicates a significant difference between groups with $p < 0.05$.

We found that GO exposure at the concentrations of 0.04 and 1 % led to lower glycogen levels in males by 30 % and 24 % as compared to control, respectively (Fig. 4C; Dunnett's test, $p < 0.05$). Similarly, 22 - 30 % decreased glycogen pool was found in females under 0.04 % and 0.2 % of GO in the diet (Fig. 4D; Dunnett's test, $p < 0.05$).

In this study, males fed by diet with GO at the concentrations of 0.04 and 0.2 % displayed ~ 35 % lower triglycerides (TAG) levels as compared to the control (Figure 4E; Dunnett's test, $p < 0.05$). Consumption of the food with GO did not affect TAG levels in females (Figure 4F).

***Effects of GO on the resistance to starvation and oxidative stress***

To investigate the effects of GO on the susceptibility to stressful conditions, we assessed the survival rates of flies reared on the media with GO under constant conditions of starvation and oxidative stress. Resistance toward starvation in males was higher under consumption of food with GO at a concentration range of 0.02-1% as compared to the control (Fig. 5A; log-rank test, $p < 0.05$). We observed increased survival under starvation conditions in females, which consumed medium with 0.02 – 1 % of GO (Fig. 5B; log-rank test, $p < 0.04$).

To further investigate the effect of GO on stress resistance, we treated flies with 20 mM menadione shown to induce oxidative stress conditions. Males reared on the media with 0.02 - 0.2 % and 1 % of GO were more resistant to oxidative stress as compared to control (Fig. 5C; log-rank test, $p < 0.03$). Survival under menadione treatment of females that consumed food with 0.02 - 0.4 % of GO was higher as compared to control (Fig. 5D; log-rank test, $p < 0.03$).

## III. Discussion

The current study focuses on checking the biological effect of GO in low doses using the *Drosophila* model. It was previously reported in mice, rabbits, and nematode worms that GO is non-toxic up to 100 - 300 mg/L [22]. Long-term exposure to low concentrations of GO showed significant beneficial effects on *Drosophila* during development, including enhanced developmental rate and pupation height. Our study is in good agreement with the study of Wang and colleagues, which demonstrated an increased pupation rate of Asian corn borer with increasing GO concentration in the food [23]. Moreover, higher larvae weight and pupal weight were found under GO exposure [23]. However, most of the reports indicated negative or no effect of different GO treatments on insects. In the relatively higher concentrations of the GO (500 and 1000 µg/ml, which correspond to 50 -100 % of GO), significant delays were observed in the pupation rate, associated with a lower percentage of flies pupated [24]. Graphene oxide may be used as a multifunctional synergist of insecticides against Lepidopteran insects [25], whereas GO can cause damage to the cement layer of insects, resulting in a rapid water loss of the insects.

Significantly shorter larval development time of GO-treated flies may be associated with up-regulated trypsin-like serine protease in both transcriptome and protein

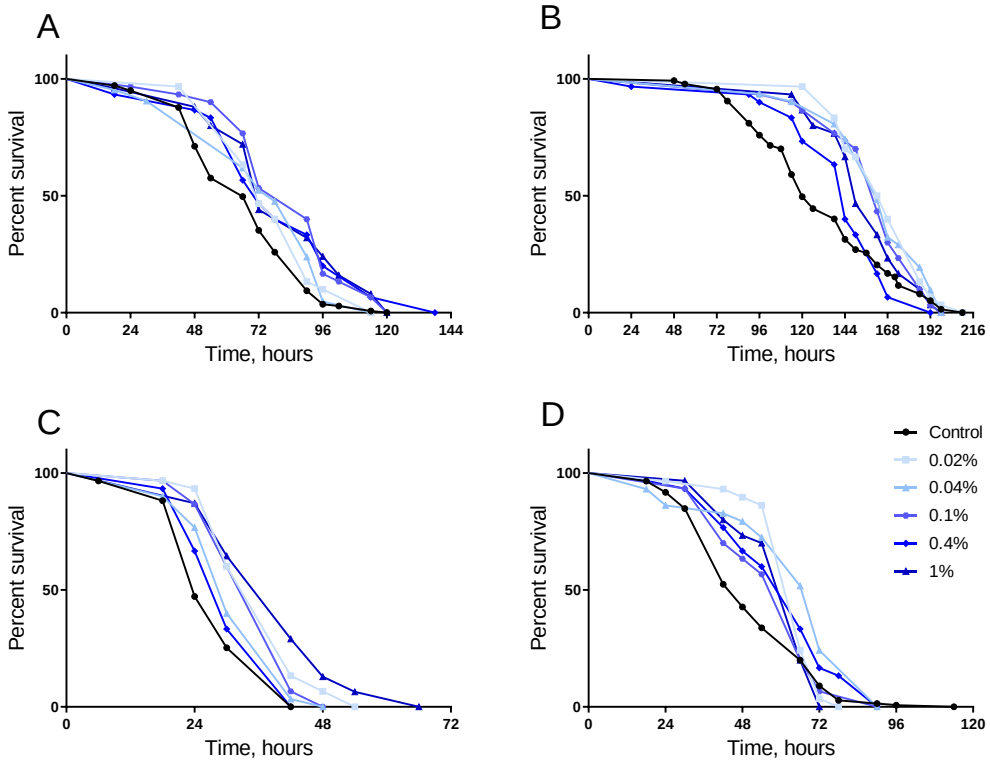


**Fig. 5.** Resistance to starvation (A – males; B – females) and oxidative stress (C – males; D – females) of flies exposed to GO. Each curve shows the fraction of individuals alive as a function of time with about 50 flies per group. Statistical analysis of differences in survival was conducted with a log-rank test.

expression levels was documented in the study by Wang and colleagues [23]. Trypsin-like serine protease is involved in the break down the dietary protein molecules into essential amino acids and peptides, which, in turn, increases nutrient adsorption and larvae growth and development [23].

In our study, a low dose of GO (0.02 - 1 %) showed beneficial effects on the physiology of flies. Lifespan, fecundity, resistance to starvation and oxidative stress were improved by long-term oral administration of GO. These results can be explained by the dietary/calorie restriction caused by the reduction of food intake under GO exposure was previously demonstrated in the study of Lee and colleagues [26]. As reported previously, GO led to higher cholesterol levels in Asian corn borer [23]. In *Drosophila*, raising larvae on a cholesterol-containing diet resulted in significantly improved stress resistance [27]. Higher cholesterol content in GO-treated *Drosophila* could contribute to the higher stress resistance that we observed in our study.

In this study, we provide several lines of evidence to prove the role of GO in carbohydrates and lipids metabolism. Firstly, GO exposure decreased glucose and glycogen levels in flies of both sexes. Secondly, consumption of the media with GO resulted in a decreased TAG level in males. In Asian corn borer, GO significantly affects the digestibility of larval food and accordingly increased the efficiency of food utilization [23]. Moreover, it was suggested that GO could be digested and metabolized, and, in turn, regulate the expression of digestion-related genes in Asian corn borer [23]. The up-regulated genes, including trypsin-like serine protease, cytochrome P450s, glutathione-*S*-transferase enzyme, small heat shock protein, chemosensory protein 1 and fatty acid-binding protein 1contribute greatly to the defensive or adaptive reaction in insects [23]. Altogether, these events improve resistance to environmental stresses.

The biodegradation of materials may play an important role in the determination of their toxicity. Materials need to biodegrade to be considered safe, as the build-up of foreign materials can lead to ecological damage and health problems. The study from Health and Environment work package showed that graphene can be degraded by human neutrophil myeloperoxidase [28]. Additional evidence of biodegradation of GO in the gastrointestinal tract using zebrafish as a model was also provided [29]. Consequently, graphene oxide is a biodegradable material.

The phenotypic response depends on insect species, doses and the way of GO exposure. The *D. melanogaster* strain $w^{1118}$ flies exposed to GO at the concentrations of 10 – 1000 μg/ml displayed developmental delay, reduction of adult eclosion in flies and decreased lifespan [24]. Used Another study demonstrated that 1 - 10 μg/ml of GO exposure led to remarkable weight loss, delayed development, retarded motion, and shortened lifespan of $w^{1118}$ flies [13]. Exposure to carbon nanofibers at a concentration of 1000 μg/ml reduced larval viability, adult fly lifespan, reproductive activity, climbing activity, and starvation resistance in *Canton-S* flies [26]. However, low concentration (100 μg/ml) of carbon nanofibers increased lifespan and climbing ability, coincident with stimulation of the antioxidant system [26]. We suggest that low concentrations of GO in the medium serve as mild stress while a low-dose stimulatory response was observed. The antioxidant defense system stimulation was previously documented to be the exact molecular mechanism underlying beneficial effects on lifespan and overall physiology in *Drosophila* under carbon nanofibers exposure at low concentrations [26]. However, high concentrations of carbon nanofibers showed adverse effects on development rate and a lifespan that is primarily associated with induction of oxidative stress [26].

## Conclusions

The emerging evidence supporting the occurrence of physiological and metabolic effects in *Drosophila* offers a relatively inexpensive high-throughput system for studies of GO toxicity or benefit on a wide range of traits. Our findings can be a useful avenue for further studies to explore the molecular mechanisms of GO action. In conclusion, the data showed that systemic administration of low doses of GO provides beneficial effects on lifespan, stress resistance, reproduction and metabolism. Our data provide additional evidence for the hormetic effect of GO treatment. Hormetic biphasic dose-response assessment may contribute to a general pattern of biological responsiveness, and can be considered a significant toxicological evaluation and have numerous biomedical applications.

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# Оцінка біологічних ефектів оксиду графену у *Drosophila*

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Графен та його похідні викликали значний інтерес дослідників через свої унікальні фізичні та хімічні властивості. Зростання комерційного виробництва супроводжується поширенням графену в навколишньому середовищі і може становити велику загрозу для багатьох живих організмів, в тому числі і для людей. У цьому дослідженні оцінили біологічний вплив оксиду графену (ОГ) у складі харчового раціону у низьких концентраціях на личинки та імаго *Drosophila melanogaster*. Споживання ОГ в концентраціях 0,02-1% підвищує швидкість розвитку личинок. Тривале застосування ОГ продовжує тривалість життя дрозофіли та значно підвищує стійкість до стресових факторів. Ми також виявили зниження рівня глюкози у гемолімфі, глікогену та триацилгліцеролів при споживанні середовищ із ОГ. Це свідчить про те, що ОГ впливає на обмін вуглеводів та ліпідів у дорослих дрозофіл. Ці висновки можуть стати корисними для оцінки біологічних ефектів ОГ для живих організмів, які можуть відігравати важливу роль у різноманітних біомедичних застосуваннях на основі графену.

**Ключові слова**: оксид графену, дрозофіла, окислювальний стрес, метаболізм, тривалість життя.