Effects of Sodium Cyclohexyl Sulfamate on Growth and Development of *Drosophila melanogaster*

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Abstract [**Objectives**] This study was conducted to investigate the effects of different concentrations of sodium cyclohexyl sulfamate on the growth and development of *Drosophila melanogaster*. [**Methods**] Different concentrations of sodium cyclohexyl sulfamate were added to the culture medium, and the effects of different concentrations of sodium cyclohexyl sulfamate on the development time and weight of *D. melanogaster* in various life stages were statistically analyzed. [**Results**] High concentration of sodium cyclohexyl sulfamate delayed the time of pupation and eclosion of *D. melanogaster*, which made *D. melanogaster* lose weight. The number of male *D. melanogaster* in the first generation was much larger than that of female individuals, which indicated that the effect of sodium cyclohexyl sulfamate on male *D. melanogaster* was greater than that of female individuals. In a word, high concentration of sodium cyclohexyl sulfamate significantly inhibited the growth and development of *D. melanogaster*. [**Conclusions**] This study provides some reference data for the research perspective of food additives and the safe use of sodium cyclohexyl sulfamate.

Key words *Drosophila melanogaster*; Sodium cyclohexyl sulfamate; Pupation; Eclosion

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Sodium cyclohexyl sulfamate is a non-nutritive synthetic artificial sweetener produced by the reaction of sulfamic acid with cyclohexylamine ($C_6H_{11}NH_2$) and NaOH. It is a kind of white needle-like and flaky crystal, odorless and easily soluble in water. Synthetic sweeteners are widely used in food industry because of their high sweetness, low energy and non-nutritive[1-2], so as to reduce the burden of diabetic patients and meet the needs of dieters[3]. Sodium cyclohexyl sulfamate is 30 times sweeter than sucrose, with pure sweetness and natural flavor, and can be used instead of sucrose or in combination with other sweeteners[4].

Because artificial sweeteners can't cause the increase of blood sugar and insulin response, food intake increases. In animal experiments, the calorie intake of rats given artificial sweeteners increased steadily after a period of time, leading to weight gain and even obesity[5-7]. A national health and nutrition survey for teenagers shows that the intake of sugary drinks is linearly and positively correlated with blood pressure[8-9]. The study conducted by Mohammed et al. showed that the systolic and diastolic blood pressure of healthy and diabetes patients who took the mixture of saccharin and sodium cyclohexyl sulfamate slightly increased, but not significantly increased, compared with patients who did not take the same mixture of saccharin and sodium cyclohexyl sulfamate. Meanwhile, in healthy individuals and diabetic patients, long-term intake of saccharin and sodium cyclohexyl sulfamate mixture could induce oxidative stress. Eating these artificial sweeteners will reduce blood sugar control, increase the risk of atherosclerosis, and also lead to impaired liver and kidney function. Moreover, increasing the daily dosage and duration of sweeteners will aggravate these effects[10]. Injection of sodium cyclohexyl sulfamate at 60 mg/kg or aspartame at 14 mg/kg to rats on the 10th to 14th day of pregnancy led to the loss of placental and fetal weight and the shortening of umbilical cord length[11].

Food safety has always been a major issue of widespread concern all over the world, which is directly related to human life and health and affects people's quality of life. The results of a national special sampling inspection by the General Administration of Quality Supervision, Inspection and Quarantine showed that among the 1 714 kinds of samples, 64 were unqualified, accounting for 3.73%. Among them, 14 kinds of samples showed sodium cyclohexyl sulfamate exceeding the standard, accounting for 21.9% of the unqualified rate, ranking first[12]. It can be seen that the safe use of sodium cyclohexyl sulfamate is particularly prominent. In order to ensure food safety, China has strictly limited the amount of sodium cyclohexyl sulfamate in food processing[13].

Drosophila melanogaster is one of the most deeply studied model organisms in biology, and its complete life cycle is divided into four distinct periods, namely, egg, larva, pupa and adult. Adult *D. melanogaster* can mate 8 h after hatching and lay eggs 2 d later. Studies have shown that the development of *D. melanogaster* is controlled by both hormones and nutrition. Extracellular signals such as nutrition determine the growth rate of *D. melanogaster*, while intracellular signals such as hormones determine the growth cycle of *D. melanogaster*, and their synergistic effect finally affects the individual size of *D. melanogaster*[14]. The time required for *D. melanogaster* to pupate and emerge into adults is an effective index for measuring the effects of environment or food components on *D. melanogaster* growth. Because many genes in *D. melanogaster* genome are evolutionarily conservative and have

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high homology with human genes[15], it has important reference significance for human health research.

In this study, different concentrations of sodium cyclohexyl sulfamate were added to the culture medium, and the effects of different concentrations of sodium cyclohexyl sulfamate on the time required for the development of *D. melanogaster* in various life stages and their weight were statistically analyzed, so as to infer some possible harm of sodium cyclohexyl sulfamate to human body and provide some reference data for the research perspective of food additives and the safe use of sodium cyclohexyl sulfamate.

Materials and Methods

Experimental materials and instruments

Experimental materials *D. melanogaster*.

Main instruments and equipment Electronic balance (Shanghai Tianping Instrument Factory, FA2004N); *D. melanogaster* incubator (Beijing Luxi Technology Co., Ltd., GYX-150FPY); stereomicroscope (Motic China Group Co., Ltd.; SMZ-161).

Experimental methods

Preparation of culture medium Sodium cyclohexyl sulfamate (Henan Midaner Trading Co., Ltd., purity 98% – 101%) was added to corn flour culture medium (agar 3 g, corn flour 40 g, sucrose 30 g, yeast 4 g, propionic acid 2 ml, distilled water), and the concentration was set to 0%, 0.035%, 0.065%, 0.085% and 0.100% respectively.

Male and female sorting of *D. melanogaster* There are obvious differences between male and female *D. melanogaster* in some morphological structures, which can be observed by a magnifying glass or stereomicroscope. Female individuals are generally larger than male individuals, and the abdomen of female *D. melanogaster* is oval in shape, while the abdomen of male *D. melanogaster* is relatively narrow and rounded. Male *D. melanogaster* have a black mane structure at the tarsal base of the first pair of feet, which looks like a small comb, that is, a sex comb, but female *D. melanogaster* do not have this structure[16].

Culture of *D. melanogaster* When raised at 25 ℃, the humidity of the incubator was 40% and the photoperiod was 12 h.

Anesthesia and observation of *D. melanogaster* In this experiment, the inverted bottle method was applied to anesthetize *D. melanogaster*[16]. When *D. melanogaster* no longer crawled, they could be observed under a stereomicroscope, and tweezers could be used to assist observation.

Measurement of growth and development indexes of *D. melanogaster* *D. melanogaster* individuals that had emerged for 8 h were placed in normal culture medium, and after growing in normal culture medium for 3 d, they were taken out and divided into groups after identifying males and females. Each group included ten pairs of *D. melanogaster*. The grouped males and females were transferred to medium containing 0.035%, 0.065%, 0.085% and 0.1% sodium cyclohexyl sulfamate, while the blank medium without sodium cyclohexyl sulfamate was used as the control group. At 24 h after egg laying, the parental *D. melanogaster* were removed.

Ten third-instar larvae were taken from each group, and the weights and pupation and eclosion time of larvae (the appearance time of the first pupa on the culture medium and the completion time of eclosion of the first pupa) in each group were observed and recorded[17]. After feeding for 3 d, taking 10 individuals as a unit, *D. melanogaster* were anesthetized by the method of inverted bottle anesthesia, and then placed on an electronic balance to measure the weight of adults, and female and male *D. melanogaster* individuals were observed under a stereomicroscope. After recording the data, they were put back into culture bottles.

Statistical analysis of data

Three groups of repeated experiments were set up for each experiment, and the experimental data were expressed by mean ± standard error ($X \pm SE$). Difference significance analysis and average linear chart analysis were carried out by using software IBM SPSS Statistics 25.

Results and Analysis

Effects of sodium cyclohexyl sulfamate on the body weight of *D. melanogaster* larvae and adults

Table 1 shows that adding different concentrations of sodium cyclohexyl sulfamate to the culture medium caused obvious effects on *D. melanogaster* larvae and adults both.

Table 1 Effects of sodium cyclohexyl sulfamate on the body weight of *D. melanogaster*

Concentration of sodium cyclohexyl sulfamate in culture medium//%	Weight of 10 third-instar larvae//g	Weight of 10 adults//g
0	0.067 9 ± 0.002 4^{a}	0.072 8 ± 0.000 1^{a}
0.035	0.037 8 ± 0.001 2^{b}	0.030 3 ± 0.000 5^{e}
0.065	0.041 5 ± 0.001 4^{b}	0.045 8 ± 0.000 2^{d}
0.085	0.023 0 ± 0.001 1^{c}	0.061 8 ± 0.000 4^{b}
0.1	0.026 6 ± 0.006 0^{c}	0.048 6 ± 0.001 3^{c}

The data were expressed by the mean ± standard error ($X \pm SE$) ($n=3$), and the significance of difference was determined by testing in software IBM SPSS Statistics 25. Different lowercase letters indicate significant differences ($P < 0.05$). The same below.

In the larval stage, the body weights of the third-instar larvae were all significantly lower than that of the control group, and the value tended to decrease with the increase of sodium cyclohexyl sulfamate concentration. In specific, there was no significant difference between concentrations 0.035% and 0.065% ($P > 0.05$), and the body weight of *D. melanogaster* decreased by 44.3% and 38.9% respectively; and there was no significant difference between concentrations 0.085% and 0.1% ($P > 0.05$), and the body weight of *D. melanogaster* decreased by 66.1% and 60.9% respectively. The weight loss increased from about 40% to 60%, and the change range reached about 50%.

In the adult stage, although the addition of sodium cyclohexyl sulfamate had a significant effect on the weight loss of adults, its influence had no obvious law. The lowest average body weight of

adult insects appeared at the sodium cyclohexyl sulfamate concentration of 0.035%, while the average body weight of *D. melanogaster* increased at concentrations 0.065% and 0.085%, and decreased again at the concentration of 0.1%. Different from the larval stage, the weight of *D. melanogaster* was also significantly different between each concentration of sodium cyclohexyl sulfamate.

As can be seen from the control group, there was no obvious difference between the weight of adult *D. melanogaster* and that of the third-instar larvae. However, when the concentration of sodium cyclohexyl sulfamate was 0.1%, the body weight of adult *D. melanogaster* increased by 82.7% compared with the third-instar larvae. When the concentration of sodium cyclohexyl sulfamate was 0.085%, the weight increased by 1.67 times. Does this imply that with the growth and development of *D. melanogaster*, the effect of sodium cyclohexyl sulfamate gradually decreases?

Effects of sodium cyclohexyl sulfamate on pupation time and eclosion time of *D. melanogaster*

As shown in Table 2, in terms of the time from egg to pupation, there were no significant differences between the 0.035% and 0.065% sodium cyclohexyl sulfamate groups and the control group, which were about 5.33 – 5.66 d, but the time for the 0.085% and 0.1% sodium cyclohexyl sulfamate groups increased to 12 – 13 d, and the differences were very significant. In terms of the time from egg to eclosion, although the 0.035% and 0.065% sodium cyclohexyl sulfamate groups were significantly different from the control group, the change was small, and the development time was slightly shortened. However, the development time of the 0.085% and 0.1% sodium cyclohexyl sulfamate groups increased to 19.67 d, and the differences were very significant. As to the time from pupation to eclosion, the experimental groups were all slightly shorter than the control group, shortened from 8.00 to 6.33 – 7.33 d, but the differences were not significant.

It could be seen that the addition of sodium cyclohexyl sulfamate had a significant effect on the whole development process of *D. melanogaster* from spawning to eclosion, but the effects of different concentrations were opposite. Low concentrations of sodium cyclohexyl sulfamate (0.035% and 0.065%) shortened the development time, while high concentrations of sodium cyclohexyl sulfamate (0.085% and 0.1%) greatly prolonged the development time. In terms of the development time from spawning to pupation, only 0.085% and 0.1% sodium cyclohexyl sulfamate caused significant differences in the development time of *D. melanogaster*. From the perspective of the development time from pupation to eclosion of *D. melanogaster*, no significant differences were observed between various sodium cyclohexyl sulfamate groups and the control group, which once again proved that high concentration of food additives has a more significant effect on larvae, but a relatively small effect on adults.

Table 2 Effects of sodium cyclohexyl sulfamate on pupation time and eclosion time of *D. melanogaster*

Concentration of sodium cyclohexyl sulfamate in culture medium//%	Time from spawning to pupation development//d	Time from spawning to eclosion development//d	Time of pupa development//d
0	5.33 ± 0.58^{b}	13.33 ± 0.58^{b}	8.00 ± 1.00
0.035	5.33 ± 0.58^{b}	11.67 ± 0.58^{c}	6.33 ± 1.15
0.065	5.66 ± 0.58^{b}	$12.67 \pm 0.58b^{c}$	7.00 ± 1.00
0.085	12.33 ± 0.58^{a}	19.67 ± 0.58^{a}	7.33 ± 0.58
0.1	13.00 ± 0.00^{a}	19.67 ± 1.15^{a}	6.67 ± 1.15

Effects of sodium cyclohexyl sulfamate on female proportion in *D. melanogaster* offspring

Male and female individuals (top 30) after eclosion treated with different concentrations of sodium cyclohexyl sulfamate were counted respectively, and the experiment was repeated for three groups. The results (Table 3) showed that the number of female *D. melanogaster* in the experimental groups cultured with different concentrations of sodium cyclohexyl sulfamate was larger than that of male *D. melanogaster*, while the ratio of fmale to emale in the control group was close to 2.3 ∶ 1. Comparing the experimental groups, when the concentration of sodium cyclohexyl sulfamate was 0.035%, the difference between male and female *D. melanogaster* was the largest, while the differences among other three groups were small. As there were more female *D. melanogaster* in the experimental groups than in the control group, it was speculated that the effect of sodium cyclohexyl sulfamate on male larvae was greater than that of females, which led to the longer pupation time of male larvae in the development process, which led to the decrease of the pupation ratio of male larvae at the same time, resulting in the smaller number of male insects in statistics. Because the number of individuals was small and the statistical time was early, it is required to increase the sample size and postpone the statistical time for further study and confirmation.

Table 3 Effects of sodium cyclohexyl sulfamate on different sexes of *D. melanogaster*

Concentration of sodium cyclohexyl sulfamate in culture medium//%	Number of male *D. melanogaster*	Number of female *D. melanogaster*	Male-female ratio of the first-generation adult *D. melanogaster* (♀ ∶ ♂)
0	21	9	2.3 ∶ 1
0.035	7	23	0.3 ∶ 1
0.065	12	18	0.67 ∶ 1
0.085	11	19	0.58 ∶ 1
0.1	12	18	0.67 ∶ 1

Conclusions and Discussion

Juvenile hormone (JH) can not only regulate the molting time of insects, but also regulate the final size of insects. Mirth *et al.* studied the developmental mechanism of larval somatotype in *D. melanogaster*, whose corpora allata (CA) producing JH had been excised through gene excision. They found that the pupation size of the larvae lacking CA was smaller than that of the control larvae, because the growth rate of the larvae decreased. JH regulates the growth rate through ecdysone and insulin signaling pathways. They believe that there is a close relationship between the process of regulating developmental changes (such as adolescence and metamorphosis) and the process of regulating growth. This hypothesis provides a physiological background for following observation[14]: in humans, developmental hormones (such as androgen and estrogen) can also promote the growth of several cancers[18-19] and benign tumors (such as vascular malformation)[20]. It could be seen that the research on the development mechanism of *D. melanogaster* is of great significance for the study of human diseases[14-15].

Excessive use of some compounds can affect the growth and development of *D. melanogaster*[17, 21-24]. High concentration of diisononyl phthalate (DINP) significantly prolonged the development time of *D. melanogaster*, and the incubation time from larva to adult was 9.87% longer than that of the control[21]. It is consistent with the results of this study: the development time of the 0.085% and 0.1% sodium cyclohexyl sulfamate groups increased to 19.67 d, showing an increase of 47.6% compared with the control group. It also implies that excessive intake of sodium cyclohexyl sulfamate may affect the development of adolescents, leading to their developmental retardation. Meanwhile, newborn *D. melanogaster* were weighed by sex, and the results showed that the weight loss increased with the increase of DINP concentration. The weights of male and female *D. melanogaster* in the 1.0% DINP group decreased by 18.34% and 13.09% respectively compared with the control[21], and injecting 60 mg/kg of sodium cyclohexyl sulfamate into rats from the 10^{th} to 14^{th} day of pregnancy led to the loss of placenta and fetal weight and the shortening of umbilical cord length[11]. The results of this study also showed similar effects. In the larval stage, the body weights of the third-instar larvae were all significantly lower than that of the control group, and there was a trend of decreasing with the concentration of sodium cyclohexyl sulfamate increasing; and in the adult stage, the addition of sodium cyclohexyl sulfamate had a significant effect on the weight loss of adults. For *D. melanogaster* eclosed in the same period, the number of male *D. melanogaster* was significantly lower than that of female *D. melanogaster*, indicating that the effect of sodium cyclohexyl sulfamate on the development time of male *D. melanogaster* was greater than that of female *D. melanogaster*, which also indicated that sodium cyclohexyl sulfamate had different effects on different sexes of *D. melanogaster*. It suggests that excessive intake of sodium cyclohexyl sulfamate has a greater impact on male adolescents. Li *et al.*[22] found that adding cordycepin to the culture medium could prolong the life span of *D. melanogaster*, and the effect of prolonging the life span for male *D. melanogaster* was better than that for female *D. melanogaster*. Feeding *D. melanogaster* with salbutamol and Chinese redbud flower honey promotes the protective mechanism of antioxidant system in vivo, which makes the life span of *D. melanogaster* treated at a certain concentration increase[17,23]. Benzoic acid also has obvious inhibitory effect on the growth and development of *D. melanogaster*. With the increase of benzoic acid concentration, the pupation time and eclosion time of *D. melanogaster* are prolonged and the weight is reduced, which is consistent with the results of this study[24]. Interestingly, the addition of Chinese redbud flower honey can also increase the fresh weight of adult *D. melanogaster* and shorten the development cycle[23], which is exactly opposite to our research results.

Very low concentration (0.06 mM) of sodium cyclohexyl sulfamate can bend and fold the microfilaments and microtubules of osteoblasts. The increase of sodium cyclohexyl sulfamate content led to a significant decrease in cell viability. Sodium cyclohexyl sulfamate can obviously inhibit the expression of bone morphogenetic protein-2 (BMP-2). Alizarin red staining experiments showed that sodium cyclohexyl sulfamate reduced the mineralization ability of osteoblasts[25]. First of all, sodium cyclohexyl sulfamate may bind to serum protein[26], which will inhibit the proliferation and differentiation of osteoblasts. Secondly, sodium cyclohexyl sulfamate can bind calcium ions, which may interrupt some signal pathways that regulate the expression of BMP2[27]. Chen *et al.*[25] insist that although sodium cyclohexyl sulfamate is cheap and convenient as a food additive, it is imperative to ban the use of sodium cyclohexyl sulfamate worldwide for the sake of people's bone health.

As a common food additive, sodium cyclohexyl sulfamate only acted as an additive in the culture of *D. melanogaster* in this experiment, and was not used as a component of the culture medium itself[22]. It is impossible to know how much sodium cyclohexyl sulfamate *D. melanogaster* eat. In this study, excessive sodium cyclohexyl sulfamate had obvious influence on *D. melanogaster*. It not only led to the weight loss of *D. melanogaster*, but also prolonged the time of pupation and eclosion, that is to say, sodium cyclohexyl sulfamate had obvious inhibitory effect on the growth and development of *D. melanogaster*. Therefore, excessive intake of sodium cyclohexyl sulfamate may be harmful to the growth and development of human body, especially the growth and development of male adolescents, and there may be phenotypic changes and gene mutations, affecting gene expression and other issues[21,25,28]. In this sense, although sodium cyclohexyl sulfamate is cheap and convenient as a food additive, for the sake of human health, the addition of sodium cyclohexyl sulfamate in processed food must be strictly monitored, especially in children's food.

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