

Effects of Acute Sodium Nitrite and Sodium Nitrate on the Development of *Drosophila melanogaster*

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Abstract: Sodium nitrite (NaNO₂) and sodium nitrate (NaNO₃) are used as food preservatives. In addition to teratogenic, mutagenic, and carcinogenic effects, these products are also thought to have endocrine disrupting effects. This study aimed to investigate the effects of sodium nitrite (NaNO₂) and sodium nitrate (NaNO₃) on the development of *Drosophila melanogaster*. Third instar larvae of Canton S strain (wild type) were treated to 25 mM, 50 mM, 75 mM NaNO₂, and NaNO₃ for 6 hours. The pupation and maturation percentages and times were determined. There was no significant difference in the percentage of pupation and maturation compared to the control group (p>0.05). However, 50 and 75 mM NaNO₂ exposures caused developmental delay in both the mean pupation and the mean maturation times (p<0.05). Additionally, it was found that mean maturation time was delayed with 50 and 75 mM NaNO₃ exposures (p<0.05).

Keywords: Food additives, *Drosophila*, pupation, maturation, developmental time delay.

Akut Sodyum Nitrat ve Sodyum Nitrit Uygulamasının *Drosophila melanogaster*'in Gelişimi Üzerine Etkileri

Öz: Sodyum nitrit (NaNO₂) ve sodyum nitrat (NaNO₃) gıda koruyucusu olarak kullanılmaktadır. Teratojenik, mutagenik ve kanserojen etkilere ek olarak, bu ürünlerin endokrin bozucu etkileri de vardır. Bu çalışmada, sodyum nitrit (NaNO₂) ve sodyum nitratın (NaNO₃) *Drosophila melanogaster* gelişimi üzerindeki etkileri araştırılmıştır. Yabani tip Canton S soyunun üçüncü evre larvalarına, 6 saat boyunca 25 mM, 50 mM, 75 mM NaNO₂ ve NaNO₃ uygulanmıştır. Pupaşma ve erginleşme yüzdeleri ve süreleri belirlenmiştir. Pupaşma ve erginleşme yüzdelerinde kontrol grubuna göre istatistiksel olarak anlamlı bir fark saptanmamıştır (p>0.05). Ancak, 50 ve 75 mM NaNO₂ uygulamaları hem ortalama pupaşma hem de ortalama erginleşme sürelerinde gelişimsel gecikmeye neden olmuştur (p<0.05). Ayrıca ortalama erginleşme süresinin 50 ve 75 mM NaNO₃ uygulaması ile ertelendiği saptanmıştır (p<0.05).

Anahtar kelimeler: Gıda katkı maddeleri, *Drosophila*, pupaşma, erginleşme, gelişim zamanı gecikmesi.

1. Introduction

Natural or synthetic food additives are substances added to almost all foods. Coloring agents, preservatives, flavors, emulsifiers, and stabilizers are the most well-known food additives (Helal, Zahkok, Soliman, Al-Kassas, & Wahed, 2008). Sodium and potassium salts of nitrate and nitrite are widely used in meat products as they contribute to the development of the characteristic color and flavor, control lipid oxidation and antimicrobial effect on pathogenic microorganisms, especially *Clostridium botulinum* that produces a deadly neurotoxin causing food poisoning (Özdestan & Üren 2010; Turp & Sucu 2016; Basak, Chatterjee, Ghosh, & Chakravarty, 2017). Contrary to the beneficial applications of these salts, excessive use in the human diet can cause health-damaging effects (Ikemoto, Teraguchi, & Kobayashi, 2002; Ansari, Ali, & Mahmood, 2015). It is also reported that nitrate and nitrite cause developmental defects (Griffis-Kyle, 2007; Hannas, Das, Li, & LeBlanc, 2010; Simmons, Karimi, Talwar, & Simmons, 2012).

The most commonly used food additives, sodium nitrite and sodium nitrate, have been tested for different biological organisms (Sarıkaya & Cakır, 2005; Sarıkaya, Cakır, & Solak, 2006; Helal, et al., 2008; Özdestan & Üren

2010; Ansari et al., 2015; Basak et al., 2017). Suzuki, Iijima, Scobie, and McColl (2005) found out that dietary nitrates are metabolized to nitrite after absorption from the intestine. In the acidic content of the stomach, it was determined that these nitrites react with amines and amides to form nitroso compounds. It was determined that nitrites and nitrosamines increased the amount of ROS (reactive oxygen species) in human hepatoma cells. Increased ROS levels are known to induce oxidative damage. In other studies, nitrite, nitrate, and nitroso-compounds have been reported to be associated with developmental disorders (Brender et al., 2011; Simmons et al., 2012). These studies have shown that nitrite and nitrates may have effects on development and directed us to work with these chemicals.

In the present study, the effects of sodium nitrite (NaNO₂) and sodium nitrate (NaNO₃) on the development of *Drosophila melanogaster* were examined considering the changes in the developmental stages. *D. melanogaster* is a dipteran insect that is often preferred in biological studies due to its short life, well-defined genetics, easy cultivation in the laboratory environment, and its genetic similarity to mammals. Thus, this organism is used for the detection of mutagenic, morphological, and developmental effects of

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different chemical agents. In addition, *D. melanogaster* is a very useful model organism for developmental biology and toxicology studies (Basak et al., 2017). For these reasons, it is thought that the results of the experiments with *Drosophila* can be adapted to the effects of food additives in humans.

2. Material and Methods

2.1. Strain, media and environmental conditions

Fly strains used in this study were the wild-type Canton S strain of *D. melanogaster*. The flies were reared on a standard cornmeal *Drosophila medium*. They were kept in a *Drosophila* culture room at $25 \pm 1^\circ\text{C}$, with 50-60% relative humidity and in 12 hours light, 12 hours dark cycle.

Virgin female and male flies of the same age were mated in culture bottles. After 8 hours, the flies were removed from the bottles and the third instar larvae were collected 72 ± 4 hours later.

2.2. Chemicals and treatment procedure

The test solutions were prepared by using solid compounds of sodium nitrite (NaNO_2) and sodium nitrate (NaNO_3). Sodium nitrite (CAS No: 7632-00-0, 99.5% purity) was obtained from Carlo Erba (Italy). Sodium nitrate (CAS No. 7631-99-4, 99% purity) was supplied from Sigma (USA). The amounts of NaNO_2 and NaNO_3 to be tested were weighed and dissolved in 1 liter of 5% sucrose (Merck; Darmstadt, Germany) solution to obtain required concentrations (25 mM, 50 mM and 75 mM).

In this study, Canton S third instar larvae of *D. melanogaster* were treated to 25 mM, 50 mM, and 75 mM NaNO_2 and NaNO_3 for six hours acute exposure. During the nitrite and nitrate exposures, larvae were placed in glass tubes (2.5'7.5 cm) containing filter papers that had absorbed 100 μL stock solutions. In this way, larvae were exposed to these chemicals by nutrition as well as by absorption from the skin. While choosing the dose, the results of previous studies were investigated (Sarıkaya & Cakır, 2005; Sarıkaya et al., 2006). Moreover, the effects of NaNO_2 and NaNO_3 on the survival of 72-hour larvae of *D. melanogaster* were investigated in the dissertation studies conducted by Özcan (2011). Therefore, the most appropriate doses were selected based on the available literature findings and the results of previous experiments. Doses of 25 mM, 50 mM, and 75 mM were selected to investigate the effects of these chemicals on the development of *D. melanogaster*.

2.3. Developmental stages observations

NaNO_2 and NaNO_3 exposed and non-exposed (control groups) larvae were placed in glass tubes (2.5'7.5 cm) that contained a standard *D. medium*. Every tube was contained ten larvae. The development of all experimental groups was followed at 4-hour intervals. The numbers and transition times of individuals passing from larvae to pupae and from pupae to adults were recorded.

2.4. Statistical analysis

The statistical analyses were applied by using the Statistical Package for the Social Sciences (SPSS) 15 program. Analysis of variance (ANOVA) test was used to determine the significance of the pupation and maturation percentages. The comparison of the pupation and

maturation times was done by a two-variable t-test. The significance level was $p < 0.05$ for all analyzes.

3. Results

3.1. Effect of the sodium nitrite (NaNO_2) and sodium nitrate (NaNO_3) on the transition percentages from larvae to pupae

Pupae developed from NaNO_2 and NaNO_3 exposed and control larvae were counted separately and their pupation percentages were detected. The statistical (one-way analysis of variance) analyses showed that nitrite and nitrate applications did not cause any statistical difference in pupation percentages ($p > 0.05$) (Table 1).

Table 1. The changes of pupation percentages depending on NaNO_2 and NaNO_3 exposures

Treatments	NaNO_2 group			NaNO_3 group		
	Rate \pm S.E.	S.D.	p	Rate \pm S.E.	S.D.	p
Control	95 \pm 0.02	0.071	0.625	95 \pm 0.02	0.071	0.814
25mM	95 \pm 0.02	0.053		97 \pm 0.02	0.067	
50mM	95 \pm 0.02	0.053		97 \pm 0.01	0.048	
75mM	91 \pm 0.04	0.129		95 \pm 0.02	0.070	

S.E.: Standard error, S.D.: Standard deviation, $p > 0.05$

3.2. Effects of the NaNO_2 and NaNO_3 on the mean pupation time

In this study, the mean pupation time for NaNO_2 and NaNO_3 at different concentrations was also determined. The mean pupation times in the experimental and control groups were compared with a two-variable t-test and the results are given in Table 2. In the control group, the mean pupation time was 52.17 hours. However, the mean pupation time increased to 52.90 and 54.41 hours in the 50 mM and 75 mM NaNO_2 treatment groups, respectively. These increases were statistically significant ($p < 0.05$). It can be said that the application of 50 and 75 mM NaNO_2 exposures cause a developmental delay by extending the transition period from larvae to pupae. In contrast to these findings, no statistically significant change in mean pupation time was observed in NaNO_3 exposure groups compared to the control ($p > 0.05$) (Table 2).

Table 2. Mean pupation times of control and NaNO_2 and NaNO_3 exposure groups

Treatment	Mean Pupation Time (hour)	
	NaNO_2 group	NaNO_3 group
Control	52.17	52.17
25 mM	52.05	51.87
50 mM	52.90*	52.83
75 mM	54.41*	52.85

*: Indicates significant delay in pupation time compared to control ($p < 0.05$)

3.3. Effect of the sodium nitrite (NaNO_2) and sodium nitrate (NaNO_3) on the transition percentages from pupae to adult

The transition rates of NaNO_2 and NaNO_3 exposure groups and control group from pupae to adults were determined and then compared by using one-way ANOVA. The results of the variance analysis showed that nitrite and nitrate applications did not cause statistically significant differences in maturation percentages compared to the control group ($p > 0.05$) (Table 3).

Table 3. The changes of maturation percentages depending on NaNO₂ and NaNO₃ exposures

Treatments	NaNO ₂ group			NaNO ₃ group		
	Rate±S.E.	S.D.	p	Rate±S.E.	S.D.	p
Control	86.9±0.04	1.117	0.467	86.9±0.04	0.071	0.796
25mM	90.5±0.03	0.078		88.9±0.03	0.099	
50mM	91.5±0.03	0.083		88.7±0.04	0.125	
75mM	93.2±0.01	0.078		91.8±0.03	0.095	

S.E.: Standard error, S.D.: Standard deviation, p>0.05

3.4. Effects of the NaNO₂ and NaNO₃ on the mean maturation time

The times to transition from pupae to adults were also followed at 4-hour intervals and the mean maturation times were determined for all groups. The mean maturation times in the experimental and control groups were compared with a two-variable t-test and the results are given in Table 4. The mean maturation time of the control group was found to be 70.92 hours. However, there were statistically significant increases in the mean maturation times of 50 mM and 75 mM exposure groups (p<0.05) but it was determined that hour changes in 25 mM nitrite and nitrate treatment groups were not statistically significant (p>0.05) (Table 4).

Table 4. Mean maturation times of control and NaNO₂ and NaNO₃ exposure groups

Treatment	Mean Maturation Time (hour)	
	NaNO ₂ group	NaNO ₃ group
Control	70.92	70.92
25 mM	71.93	71.18
50 mM	73.65*	72.82*
75 mM	76.24*	73.66*

*: Indicates significant delay in maturation time compared to control (p<0.05)

4. Discussion

We chose sodium nitrite (NaNO₂) and sodium nitrate (NaNO₃) for our study. They are commonly used in meat as a food coloring and flavoring agent. Nitrites and nitrates also prevent the growth of *Clostridium botulinum* and the formation of its toxins (Sarıkaya & Cakır, 2005). In our experiments, it was observed that sodium nitrite and sodium nitrate applications did not affect the pupation and maturation percentages but the mean pupation time was delayed in 50 mM and 75 mM NaNO₂ exposure groups (p<0.05). Besides, the maturation time was prolonged in 50 mM, 75 mM NaNO₂ and NaNO₃ exposure groups (p<0.05).

Basak et al. (2017) investigated the developmental effects of sodium and potassium salts of nitrite and nitrate by using *Drosophila melanogaster*. The concentration of 40 mM, NaNO₂ and KNO₂ stopped *Drosophila* development completely. In 20 and 30 mM NaNO₂ and KNO₂ exposure groups, the developmental delay was observed. However, NaNO₃ and KNO₃ exposures did not lead to any significant changes compared to control in the developmental outcomes of *Drosophila*. In another study, Sarıkaya et al. (2006) investigated the effect of NaNO₂, NaNO₃, KNO₂ and KNO₃ on the life span of *D. melanogaster*. 75mM doses of these substances were found to be statistically significant reduction in the life span compared to the control group. Also, Sarıkaya et al. (2006) found that the effect of nitrite was higher than nitrate.

Cheng and Chen (2002) conducted a study with the Asian tiger shrimp *Panaeus monodon* and applied NaNO₂ and NaNO₃ to these organisms. They investigated the accumulation of nitrite and nitrate in the tissues. Cheng and Chen (2002) found out that nitrite and nitrate have similar toxicological mechanisms but nitrite accumulates in the tissues more than nitrate. In addition, it is stated that organisms convert nitrate to nitrite and toxicity occurs in this way. In the present study, NaNO₂ was effective on both pupation and maturation times whereas NaNO₃ only delayed maturation time. This may be due to the fact that nitrate given by feeding is converted to nitrite in *D. melanogaster's* tissues and shows its effect in this way. Therefore, the effect of nitrate on development may have emerged at the time of maturation.

Siikavuopio and Sæther (2006) investigated the effects of nitrite concentration on growth in juvenile cod, *Gadus morhua*. Significantly reduced growth was found in fish exposed to all treatment levels during later periods. Similarly, Balangoda, Deepananda, and Wegiriya (2018) examined the potentially toxic effects of environmentally relevant nitrate concentrations on the development, growth, and mortality of early life stages of common hour-glass tree frog, *Polypedates cruciger*. No behavioral changes or abnormalities due to nitrate exposure were reported in the control and nitrate-induced group. However, it was found out that nitrate exposures significantly increased (p<0.05) the growth of the tadpoles up to 25 days old.

Guillette and Edwards (2005) examined the effect of NO_x. It was stated that these compounds may be disrupting the reproductive physiology of wildlife. They suggested that the toxicity of NO_x may be due to binding to heme groups of steroidogenic enzymes and inhibiting hormone synthesis. Panesar and Chan (2000) proposed that NO_x converts to nitric oxide which then binds to heme groups of steroidogenic enzymes in cells and suppresses their activities. This conversion is thought to be one of the main causes of endocrine disruption.

Ecdysteroids are steroid hormones that have essential roles in the development of *D. melanogaster* (Atlı & Ünlü, 2012). Hannas et al. (2010) reported that nitric oxide acts by lowering ecdysteroid levels. They hypothesized that nitrite and nitrate are converted to nitric oxide in *Drosophila* cells and disrupt normal endocrine activities. In the same study, it was stated that nitrite was converted to nitric oxide more effectively than the nitrate and its cellular uptake was higher. In addition, it was proposed that NO₂ was more toxicologically significant than was NO₃ due to its reproductive and developmental toxicity at low exposure levels. In our study, it was found that NaNO₂ changed both pupation and maturation time but NaNO₃ only affected maturation time. It is a conclusion to support the results of Hannas et al. (2010). In our study, NaNO₂ disrupts the development more quickly and it takes longer for the NaNO₃ to take effect.

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