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ORIGINAL ARTICLE

Sodium and potassium nitrite-induced developmental genotoxicity in *Drosophila melanogaster* – effects in larval immune and brain stem cells

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ABSTRACT

This present report reveals the important aspects of sodium and potassium nitrite-induced genotoxicity during the development of a model organism, *Drosophila melanogaster.* Dietary sodium nitrite at ≥20 mM concentration caused significant nuclear deformation of two important larval cells, namely hemocytes and neuroblasts, that play important roles in immunity as well as growth and metamorphosis of the fly. In contrast, potassium nitrite induced significant nuclear deformities only in hemocytes at 30 mM concentration. Due to the unavailability of any report regarding the developmental genotoxic effects of nitrite food preservatives in immune and neural stem cells, results of our study deserve special mention as these provide first evidences of genotoxicity in these cells of the developing *Drosophila* larvae by these food additives.

KEY WORDS: DAPI; hemocytes; neuroblasts; genotoxicity

Introduction

Sodium and potassium nitrite ($NaNO_2$ and KNO_2) are widely used as food preservatives in meat preparations and in several meat products. These inhibit *Clostridium botulinum*, a gram-positive bacteria whose toxin causes a serious food poisoning, namely botulism, with a high mortality rate. Moreover, it provides color, flavor and antioxidant capacity to meat products (Sprong *et al.,* 2017). However, excessive dietary intake of nitrites pose threat to human health as they react with amines and amides in the stomach to form nitroso compounds that increase the risk of cancer (Braun *et al.,* 1977; Mirvish,1994). Nitrosamines raised the intracellular reactive oxygen species (ROS) in human culture cells and caused damage to cellular DNA indicating the genotoxic potential of $NaNO_2$ and KNO_2 (Erkekoglu & Baydar, 2010).

By using a versatile model organism, *Drosophila melanogaster* (*D. melanogaster*), Sarıkaya & Cakır, 2005 had demonstrated that $NaNO_2$ and KNO_2 were associated with developmental genotoxicity in this eukaryote. The results of our work (Basak *et al.,* 2017) published in this journal also demonstrated that the exposure of the fly to $NaNO_2$ was associated with its developmental inhibition by delaying metamorphosis and reducing the number of pupae and young adults in a dose dependent manner. The developmental inhibition of the fly was also accompanied with the increase in the number of polytene chromosomal aberrations of the larval salivary gland. As an extension of that study, in this report we had tried for the first time to demonstrate whether $NaNO_2$ and KNO_2 exerted genotoxicity or not in two important larval cells that were considered to be vital for immunological defense, cellular differentiation, growth and development of the fly (Holz *et al.,*2003; Kaplan *et al.,* 2008; Johnson *et al.,* 2018; Nanda *et al.,* 2019). As *D. melanogaster* possesses substantial genetic homology

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with human and harbors several orthologs of human disease genes (Mao *et al.,* 2018), our report envisages the possibility of similar vulnerability of dietary $NaNO_2$ during human development.

Material and methods

Chemicals

Sodium nitrite ($NaNO_2$ ≥98%, impurities: chloride ≤0.005%, sulfate ≤0.005%, lead ≤0.0005%, calcium ≤0.001%, arsenic ≤0.00004% etc., Merck, India), potassium nitrite (KNO_2 97%, impurities: chloride 0.02%, sulfate 0.02%, sodium 0.05% etc., CDH, india), sodium chloride (NaCl 99%, Merck, India), potassium chloride (KCl >99%, SRL, India), calcium chloride ($CaCl_2$. 2H2O >99% , Merck, India), di-sodium hydrogen phosphate (Na_2HPO_4 99%, SRL, India), potassium dihydrogen phosphate (KH_2PO_4 >99%, Merck, India), glacial acetic acid (CH_3COOH, Merck, India), propionic acid (CH_3CH_2COOH, Merck, India), methyl paraben (Loba-Chemie, India), ethanol (Merck, India), lactic acid ($CH_3CH(OH)CO_2H$, SRL, India), orcein (for microscopy, Loba-Chemie, India), agar, yeast (Kothari fermentation and biochem Ltd, India), n-phenylthiourea ($C_7H_8N_2S$, Central Drug House, India) and 4′,6-diamidino-2-phenylindole (DAPI) (Himedia Laboratories Pvt Ltds, India) etc. were used for this study.

Fly culture

Drosophila culture medium was prepared according to the method described by Basak *et al.* (2017). For mating, five adult male and five adult female *Drosophila melanogaster* were released in each 50 ml glass culture vial containing 10 ml of culture medium without (control) or with a specific concentration of $NaNO_2$ and KNO_2 (15 mM, 20 mM and 30 mM of each compound). Culture vials were maintained at 25 °C in a BOD (bio-oxygen demand) incubator. Mating flies were removed from each culture vial, immediately after the initial appearance a pupa. Five replications of culture, representing either control or a specific concentration of $NaNO_2$ and KNO_2 were made.

Isolation of larval hemocytes and neuroblast

Isolation of circulating hemocytes of the third instar larvae was done following the method of Basak *et al.* (2019), which was a modified version of the procedure originally reported by Sierra *et al.* (2014). Briefly, a sterilized larva was placed in 20 µl of phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 6.7 mM Na_2HPO_4 and 1.5 mM KH_2PO, pH 7.4) containing phenylthiourea on a glass slide. Hemolymph containing hemocytes was allowed to spill out of the larva by disrupting its cuticle taking care not to put injuries to the internal organs. Hemolymph of 40 larvae, either from control culture or from each of the $NaNO_2$ and KNO_2 treatment, were pooled to obtain sufficient number of hemocytes.

Isolation of neuroblasts of the third instar larvae, either from control or treatment, was done following the method of Basak *et al.* (2019), which was based on the procedures reported by Dual *et al.* (2010) and Sierra *et al.* (2014). Briefly, each third instar larva was washed very well with Ringer's solution (2 mM $CaCl_2$, 35 mM KCl, 130 mM NaCl, pH 7.2) and placed in 20 µl of the same solution on a glass slide. Holding the larva at the posterior of the midpoint by a forcep, the cuticle was ruptured behind mouth hooks with another forcep and the mouth parts were removed. The brain was detached from the surrounding tissues and transferred to a Ringer drop on paraffin film, wrapped over a glass slide. Brain lobes of five larvae of a particular group were broken, torn and shredded in the Ringer drop. Individual neuroblasts were obtained by adding 20 µl of Ringer to the brain cell suspension, followed by their pipetting up and down for several times with a micropipette.

DAPI assay

Necessary steps needed in this assay were performed following the method of Basak *et al.* (2019). Hemocytes and neuroblast preparations were centrifuged at 3000 rpm for 10 min at 4 °C. The supernatants were discarded and 20 µl of PBS (pH 7.4) was added to each precipitate. The whole content was flushed very well with a micropipette to make a cell suspension. 10 µl of aqueous DAPI solution (1 µg/ml) was added to the suspension after its transfer onto a clean glass slide. The solution mixture was mixed very well by pipetting up and down with the help of a micropipette. A cover glass was put over the solution and the slide was kept in the dark for 10 min. Visualization of the stained nuclei of hemocytes and neuroblasts was done under an inverted fluorescent microscope (Olympus (CKX53) using a software (Q imaging).

Data analyses

Assessment of nuclear deformations of larval hemocytes and neuroblasts was performed by counting the number of hemocytes or neuroblasts with abnormal nuclear morphologies out of 20 randomly selected DAPI stained cells in a single slide. Three replications of slides of each group, i.e. either control or a $NaNO_2$/KNO_2 treated group were made. Percentage (mean ± SD) of cells with deformed nuclei in a single slide belonging to control or a treatment group was calculated by the following formula:

$$\frac{\text{Number (mean±SD) of cell with deformed nuclei}}{\text{Number of total cells oserved (n=20)}} \times 100$$

Levels of differences of the percent nuclear deformities of hemocytes and neuroblasts of the larvae between the control and each test group was determined by t-test.

Results

Compared to control, DAPI assay demonstrated increased structural deterioration of the nuclear architecture of larval hemocytes (Figure 1) and neuroblasts (Figure 2) with the increasing dietary concentrations of $NaNO_2$ (Figures 3

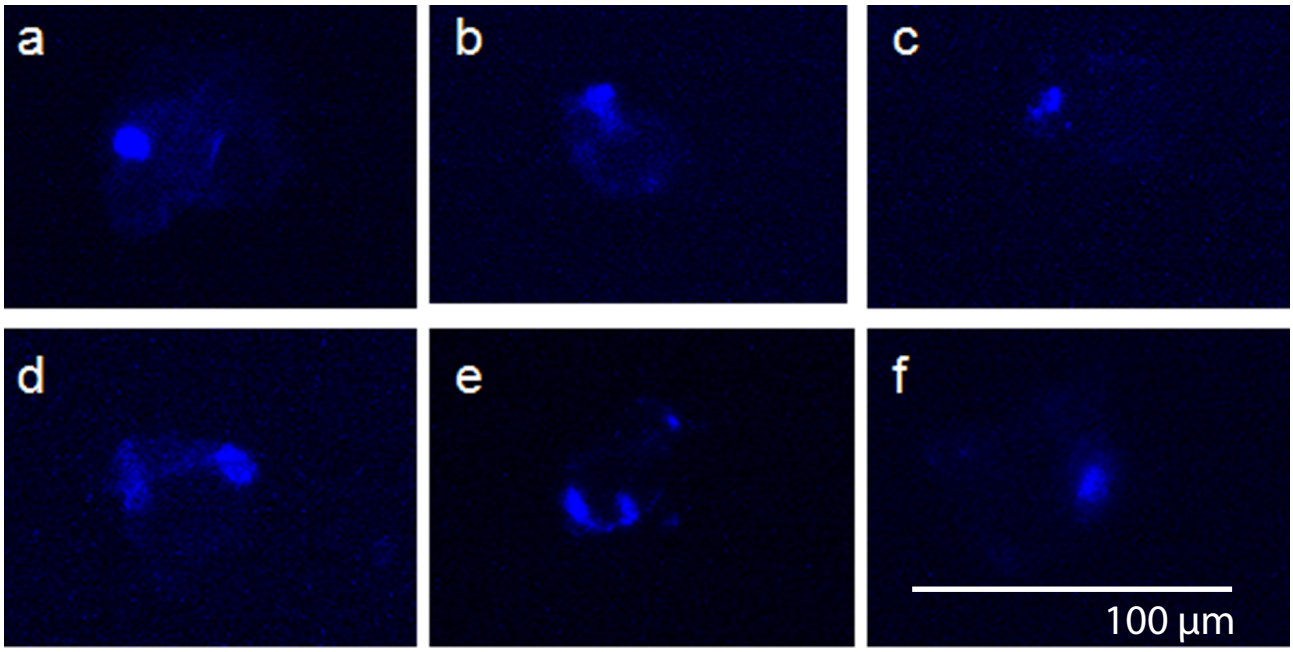

Figure 1. Detection of nuclear morphological abnormalities of hemocytes by DAPI assay. **a** – control showing round-shaped nucleus, **b** – diffused nucleus at $NaNO_2$ 20 mM, **c** – granular nucleus at $NaNO_2$ 30 mM, **d** – diffused nucleus at $NaNO_2$ 30 mM, **e** – fragmented nucleus at $NaNO_2$ 30 mM, **f** – triangular nucleus at KNO_2 30 mM.

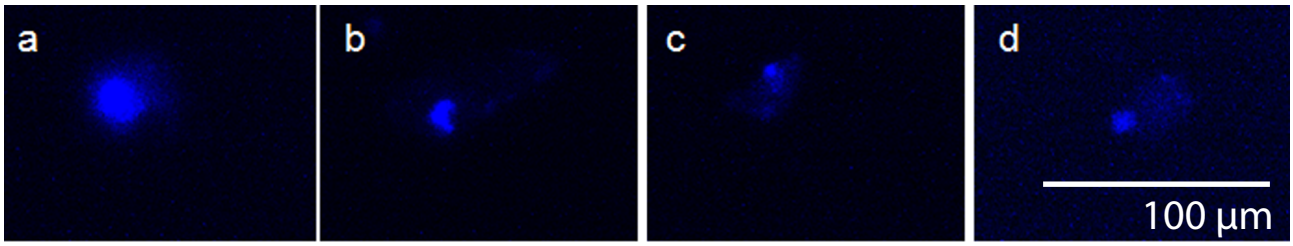

Figure 2. Detection of nuclear morphological abnormalities of neuroblasts by DAPI assay. **a** – control showing circular nucleus, **b** – hook-like nucleus at $NaNO_2$ 20 mM, **c** – granular nucleus at $NaNO_2$ 30 mM, **d** – diffused nucleus at $NaNO_2$ 30 mM.

and 4). However, significant ($^*p<0.05$, $^{**}p<0.0001$) nuclear deformations of both hemocytes and neuroblasts occurred due to exposure of developing flies to $NaNO_2$ ≥20 mM. On the other hand, KNO_2 treatment caused significant ($^*p<0.05$) nuclear deformations of hemocytes only at 30 mM concentration without rendering any significant nuclear abnormality of neuroblasts. Exposure of larvae to 30 mM $NaNO_2$ demonstrated nearly 20% higher nuclear deformations in hemocytes than those induced by KNO_2 at this concentration (Figure 3). In addition nearly 20% more nuclear deformities were induced in hemocytes by $NaNO_2$ at 30 mM than in the neuroblasts at the same concentration of this food additive (Figures 3 and 4).

In contrast to general spherical nuclear appearances of hemocytes (Figure 1a) and neuroblasts (Figure 2a) in control, nuclei of these cells in treated larvae underwent deformities and exhibited various defective shapes such as diffused (Figures 1b, d and 2d), fragmented (Figure 1e), hook-like structure (Figure 2b), granular (Figures 1c, 2c), triangular (Figure 1f) *etc.*

Discussion

In this report, we have shown the genotoxic influence of $NaNO_2$ and KNO_2 in two important larval cell types of *D. melanogaster*, which are used as tools in assessing the genotoxic potential of various chemicals (Sierra *et al.* 2014). These two cells, namely circulating hemocytes and neuroblasts, enable the larvae to combat infections and promote their growth and development (Holz *et al.,* 2003; Kaplan *et al.,* 2008; Johnson *et al.,* 2018; Nanda *et al.,* 2019). Although dietary $NaNO_2$ and KNO_2 in this study induced dose dependent nuclear deformations of both larval hemocytes and neuroblasts, significant nuclear abnormalities in these cells were noticed under the exposures of the developing flies to $NaNO_2$ ≥20 mM. In contrast, KNO_2 only induced significant nuclear structural abnormality of hemocytes at 30 mM concentration indicating the lesser genotoxic potential of this food additive than $NaNO_2$. In a previously published article in this journal (Basak *et al.,* 2017), we had shown that dietary $NaNO_2$

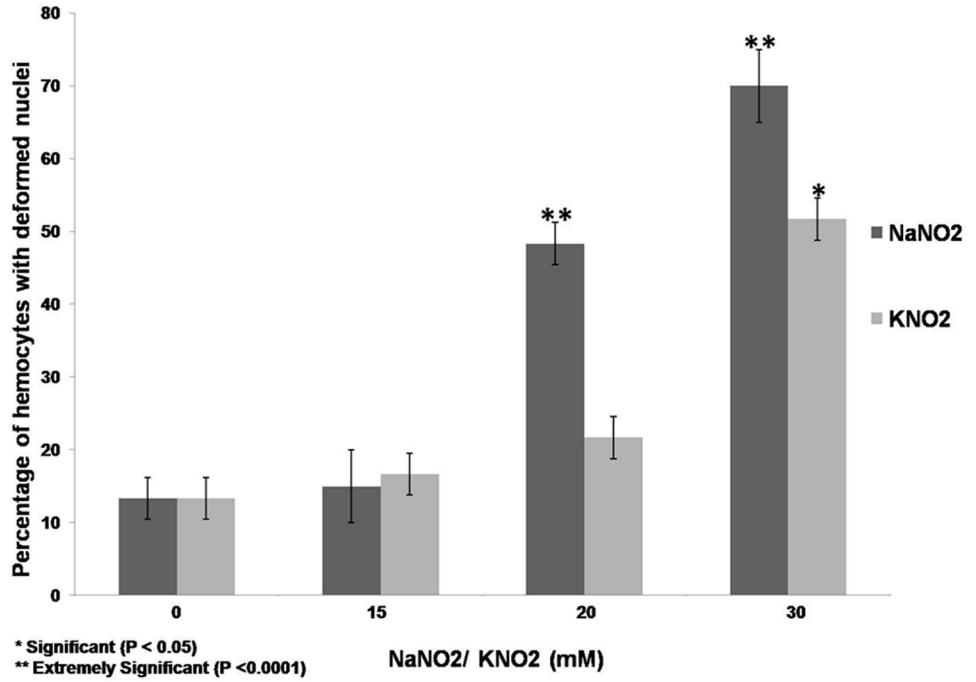

Figure 3. Percentage of hemocytes with deformed nuclei of the third instar larvae of *D. melanogaster*. It appears that dietary $NaNO_2$ caused dose-dependent increases in nuclear abnormalities of the larval hemocytes with significant changes at $NaNO_2$ ≥20 mM. KNO_2 caused significant nuclear abnormalities 30 mM only. **p*<0.05 (significant), ***p*<0.0001 (extremely significant).

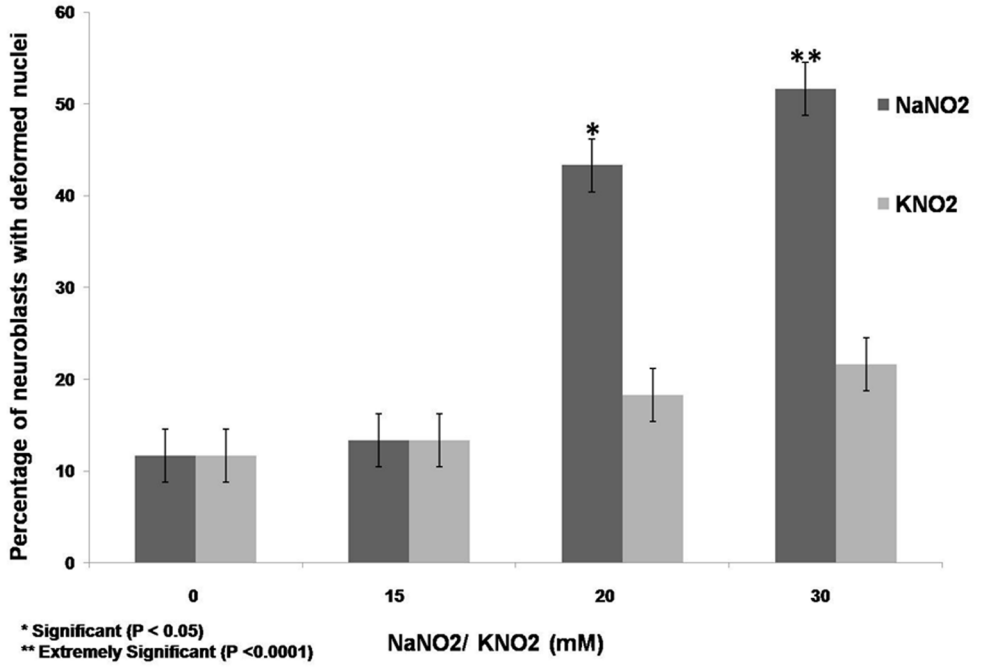

Figure 4. Percentage of neuroblasts with deformed nuclei of the third instar larvae of *D. melanogaster*. It appears that dietary $NaNO_2$ caused dose-dependent increases in nuclear abnormalities of the larval neuroblasts with significant changes at $NaNO_2$ ≥20 mM. KNO_2 failed to induce any significant nuclear deformation in larval neuroblasts. **p*<0.05 (significant), ***p*<0.0001 (extremely significant).

caused the dose dependent delay in metamorphosis as well as reduction of progeny yield of the fly in response to 10–30 mM concentration, but significant inhibitory influence was observed only at 30 mM $NaNO_2$. Moreover, KNO_2 was unable exert any genotoxic influence in the development of the fly.

Although our previous study failed to demonstrate the significant inhibitory effect of 20 mM $NaNO_2$ and any dietary concentration of KNO_2 on the development of the fly, induction of significant nuclear deformities of larval hemocytes by KNO_2 and a further lower concentration of $NaNO_2$ is alarming. In addition, documentation

of neurogenotoxic effect of $NaNO_2$ in this study implies vulnerability of one of the most vital organs *i.e.*, brain due to the dietary intake of this food additive.

Hemocytes in *Drosophila* larvae provide immunological defense by engulfing and melanizing pathogens and secreting antimicrobial peptides. Furthermore, hemocytes phagocytose the apoptotic cells and thereby promote embryonic tissue formation as well as organ remodeling in this fly during metamorphosis (Holz et al 2003; Nanda *et al.,* 2019). On the other hand, neuroblasts, the stem cells in the larval brain, release a protein nucleostemin 3(NS3) that control their body growth by regulating the insulin signaling pathway. It has been shown that ns3 mutant flies exhibited substantial developmental delay and many of them died before emerging as adults (Kaplan *et al.,* 2008; Johnson *et al.,* 2018). It seems that nuclear deformities in hemocytes and neuroblasts might damage the genes related to immunity, growth and development of the larvae. We speculate that $NaNO_2$ -induced functional disruptions of these genes might be responsible for inhibition of immune functions and growth signals that led to delayed metamorphosis and population inhibition of the developing flies in our previous report (Basak *et al.,* 2017). As the larval neuroblasts are differentiated to adult neurons (Riebli *et al.,* 2013), loss of functional larval neuroblasts may underpin the deficiencies in the development of central nervous system. Owing to extensive genetic similarities (nearly 65–80%) between *Drosophila* and human (Mao *et al.,* 2018), $NaNO_2$ and KNO_2-induced possibilities of injuries of the nuclear genetic materials during human development cannot be ruled out. The functional similarities of *Drosophila* larval hemocytes and vertebrate macrophages (Gold & Brückner, 2015) may indicate the innate immune system of the developing mammals including human as the possible target of $NaNO_2$ and KNO_2 mediated genotoxicity. Our experimental results obtained from the fly also confirmed the developmental neurogenotoxic effect of $NaNO_2$ that may be a possible factor for neural defects during mammalian development.

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REFERENCES

Basak AK, Chatterjee T, Ghosh SK, Chakravarty A. (2017). Impacts of dietary exposure to sodium or potassium salts of nitrate and nitrite on the development of Drosophila melanogaster. *Interdiscip Toxicol* **10**: 70–78.

Basak AK, Chatterjee T, Chakravarty A, Ghosh SK. (2019). Silver nanoparticle-induced developmental inhibition of *Drosophila melanogaster* accompanies disruption of genetic material of larval neural stem cells and non-neuronal cells. *Environ Monit Assess* **191**: 497.

Braun R, Schoneich J, Ziebarth D. (1977). *In vivo* formation of N-nitroso compounds and detection of their mutagenic activity in the host-mediated assay. *Cancer Res* **37**: 4572–4579.

Daul AL, Komori H, Lee CM. (2010). Immunofluorescent staining of Drosophila larval brain tissue. *Cold Spring Harb Protoc.* https://doi.org/10.1101/pdb.prot5460

Erkekoglu P and Baydar T. (2010). Evaluation of the protective effect of ascorbic acid on nitrite- and nitrosamine-induced cytotoxicity and genotoxicity in human hepatoma line. *Toxicology Mechanisms and Method* **20**: 45–52.

Gold KS and Brückner K. (2015). Macrophages and cellular immunity in *Drosophila melanogaster*. *Semin Immunol* **27**: 357–68.

Holz A, Bossinger B, Strasser T, Janning W, Klapper R. (2003). The two origins of hemocytes in Drosophila. *Development* **130**: 4955–4962.

Johnson PW, Doe CQ, Lai S. (2018). Drosophila nucleostemin 3 is required to maintain larval neuroblast proliferation. *Dev Biol* **44**: 1–12.

Kaplan DD, Zimmermann G, Suyama K, Meyer T, Scott MP. (2008). A nucleostemin family GTPase, NS3, acts in serotonergic neurons to regulate insulin signaling and control body size. *Genes Dev* **22**: 1877–1893.

Mao BH, Chen ZY, WangYJ, Yan SJ. (2018). Silver nanoparticles have lethal and sublethal adverse effects on development and longevity by inducing ROS-mediated stress responses. *Sci Rep* **8**: 2445.

Mirvish SS. (1994). Experimental evidence for inhibition of N-nitroso compound formation as a factor in the negative correlation between vitamin C consumption and the incidence of certain cancers. *Cancer Res* **54**(7 Suppl): 1948s–1951s.

Nanda KP, Kumari C, Dubey M, Firdaus H. (2019). Chronic lead (Pb) exposure results in diminished hemocyte count and increased susceptibility to bacterial infection in Drosophila melanogaster. *Chemosphere* **236**: 124349 .

Riebli N, Viktorin G, Reichert H. (2013). Early-born neurons in type II neuroblast lineages establish a larval primordium and integrate into adult circuitry during central complex development in *Drosophila*. *Neural Dev* **8**: 6.

Sarıkaya R and Cakır S. (2005).Genotoxicity testing of four food preservatives and their combinations in the *Drosophila* wing spot test. *Environ Toxicol Pharmacol* **20**: 424–30.

Sierra LM, Carmona ER, Aguado L, Marcos R. (2014). The comet assay in Drosophila: neuroblast and hemocyte cells. In *Genotoxicity and DNA Repair. Methods in Pharmacology and Toxicology (*Sierra L, Gaivão I eds) pp 269–282, Humana Press, New York

Sprong RC, Niekerk EM, Beukers MH. (2017). Intake assessment of the food additives nitrites (E 249 and E 250) and nitrates (E 251 and E 252). RIVM Letter report 2016–0208.