

ORIGINAL ARTICLE

Impacts of dietary exposure to sodium or potassium salts of nitrate and nitrite on the development of *Drosophila melanogaster*

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ABSTRACT

The effects of four food additives, namely sodium nitrite (NaNO_2), sodium nitrate (NaNO_3), potassium nitrite (KNO_2), and potassium nitrate (KNO_3), on animal development were evaluated by using *Drosophila melanogaster*, a model organism. Adult male and female flies were allowed to breed in culture medium, each containing one of 4 concentrations, i.e. 10, 20, 30 or 40 mM of the above mentioned salts. The concentration of 40 mM, NaNO_2 and KNO_2 completely arrested the development of the flies. Of the different concentrations of the four salts tested, exposure of flies to 30 mM NaNO_2 exhibited only significant delays in the initial appearances of third instar larvae, pupae and young adults, along with huge reduction in the number of pupae and young adults compared to controls. Rearrangements like inversions, deletion looping, regional shrinking, as well as highly enlarged puffing, etc. were also observed in the polytene chromosomes of the third instar larvae exposed to 30 mM NaNO_2 . Developmental outcomes of the flies exposed to varying concentrations of NaNO_3 and KNO_3 did not differ significantly from the controls. Owing to the extensive genetic homology between *Drosophila* and human and the successful uses of this fly as models in developmental and toxicological studies, we speculate that the experimental results exhibited by this organism in our study strongly advocate for abstaining from the dietary use of NaNO_2 and KNO_2 during human pregnancies to avoid possible negative developmental outcomes.

KEY WORDS: food additives; *Drosophila melanogaster*; polytene chromosomes; rearrangements; sodium nitrite

Introduction

Nitrate and nitrites are naturally available inorganic ions occurring as components of the nitrogen cycle (EFSA Scientific Opinion, 2009; Cabello & Roldán, 2004). Although nitrogen is an essential element for the growth and development of plants, excessive uses of fertilizers in agriculture increase nitrate concentrations in soil and water, rendering its accumulation in edible vegetables (Katan, 2009; Liu *et al.*, 2014; Marinov & Marinov, 2014), which is not suitable for human consumptions (Ikemoto *et al.*, 2002). Nitrate and nitrite are also used as preservatives, antimicrobial agents and color fixatives in processed foods like cured meat, poultry, fish,

cheese, etc. (Cammack *et al.*, 1999; Iammarino & Taranto, 2013; Cockburn *et al.*, 2014). Sodium or potassium salts of nitrate and nitrite are added to meat and other food products to prevent the growth of the dangerous bacteria *Clostridium botulinum* that produce deadly neurotoxin causing food poisoning (Cammack *et al.*, 1999; Benjamin & Collin J, 2003; Marianski *et al.*, 2009; Roberts *et al.*, 2000). In spite of the beneficial applications of these salts, their excessive dietary consumption causes a great threat to human health (Ikemoto *et al.*, 2002; Galaviz-Villa & Landeros-Sánchez, 2010; Ziarati & Arbabi-Bidgoli, 2014). After intestinal absorption, some dietary nitrates enter the saliva via circulation and are metabolized to nitrite (Björne, 2004; Suzuki *et al.*, 2003; Suzuki *et al.*, 2005). In the acidic milieu of the stomach, this nitrite, along with dietary nitrite, can react with amines and amides to form nitroso compounds (Suzuki *et al.*, 2003; Erkekoglu and Baydar, 2010; Huber *et al.*, 2013). Both nitrite and nitrosamines are reported to have raised levels of intracellular reactive oxygen species (ROS) in human hepatoma cell

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line, within a short period of time (Erkekoglu and & Baydar, 2010). Increased ROS level may induce oxidative damage of the cell membrane, DNA and proteins (Erkekoglu and & Baydar, 2010; Bandyopadhyay *et al.*, 1999; Sharma P *et al.*, 2012; Valko *et al.*, 2007) and may lead to many diseases including cancer (Valko, 2004). Although animal studies have indicated that nitrite and nitroso compounds may traverse the placenta (Alaoui-Jamali *et al.*, 1989; Gruener *et al.*, 1973), there is conflicting information regarding the reproductive outcomes of exposure to nitrates and nitrites and nitroso compounds. Some studies involving rodents exposed to nitrite (Globus & Samuel, 1978; Shimada, 1989) and some human studies that involved maternal consumption of nitrates, nitrites and nitrosamines (Huber *et al.*, 2013; Croen *et al.*, 2001) failed to correlate any significant association of these compounds with several types of birth defects. Contrary to these findings, one animal (Simmons *et al.*, 2012) and some human studies (Brender *et al.*, 2004; Brender *et al.*, 2011) reported that nitrate, nitrite and nitroso compounds were associated with developmental defects. These controversial reports provided us an impetus to evaluate some aspects of the reproductive outcomes of exposures to sodium or potassium salts of nitrate and nitrite in different stages of development in a dose dependent manner, on using the model organism *Drosophila melanogaster* (Beckingham *et al.*, 2005). In addition, polytene chromosomes of the third instar larvae of this fly were examined to ascertain whether developmental abnormality, if any, was accompanied with chromosomal changes.

Materials and methods

Chemicals

Sodium nitrite (NaNO_2 , Merck Specialities Private Ltd, India), sodium nitrate (NaNO_3 , Merck Specialties Private Ltd, India), potassium nitrite (KNO_2 , Loba-Chemie, India), potassium nitrate (KNO_3 , Merck Specialties Private Limited, India), agar, sodium chloride (NaCl , Sisco Research Laboratories, India), potassium chloride (NaCl , Sisco Research Laboratories, India), calcium chloride (NaCl , Sisco Research Laboratories, India), yeast (Kothari fermentation and biochem Ltd, India) glacial acetic acid (CH_3COOH , Sisco Research Laboratories, India) propionic acid ($\text{CH}_3\text{CH}_2\text{COOH}$, Sisco Research Laboratories, India), methyl paraben (methyl-p-hydroxybenzoate, Loba-Chemie, India), Ethanol (Merck Specialities Private Limited, India), Lactic acid ($\text{CH}_3\text{CH}(\text{OH})\text{CO}_2\text{H}$, Sisco Research Laboratories, India), orcein (for microscopy, Loba-Chemie, India) were used for this study.

Fly culture

Five adult male and five adult female *Drosophila melanogaster* were allowed to mate each in a 50 ml glass culture vial with 10 ml standard *Drosophila* culture medium containing agar, maize powder, molasses, yeast, propionic acid and the antimicrobial agent methyl paraben with varying concentrations (*i.e.* 10 mM, 20 mM, 30 mM and

40 mM) of NaNO_2 , KNO_2 , NaNO_3 and KNO_3 , separately. Each culture vial containing a particular salt of a particular concentration was designated as one of the several test vials. Control vials also contained five male and female mating partners and the medium included all the above mentioned ingredients except for any nitrite or nitrate salt added. Numbers of culture vials containing food medium were restricted to six for the control group as well as for each test group. All culture vials were maintained at 25 °C in a B.O.D. After the initial appearance of pupa, the breeding male and female flies were removed from the culture vials.

Preparation of polytene chromosomes of *Drosophila* for microscopic examinations

Mature third instar larvae exposed to different salts of different concentrations under our study as well as control larvae were used for the isolation of polytene chromosomes. Larvae were taken from culture vials on a grooved slide in ringer solution and a pair of salivary glands was dissected out of each larva under a dissecting microscope. Three pairs of such dissected glands were taken from third instar larvae exposed to each concentration of each salt in culture medium separately on a glass slide. Aceto-alcohol (1:3 ratio of glacial acetic acid and ethanol) was added to them drop-wise for two minutes for fixation. After fixation, the glands in slides were stained with a drop of aceto-orcein for 10–15 minutes keeping them covered under a petridish. After this the stain was carefully absorbed by a filter paper and glands were washed with one or two drops of 50% acetic acid to remove any excess stain. Excess acetic acid was absorbed out with a filter paper and 1–2 drops of lacto-orcein was added on the glands. A cover glass was mounted over the glands and the whole slide was wrapped with filter paper to remove excess mounting material. Uniform and gentle thumb pressure was applied on the cover glass above the glands to squash them for proper spreading of polytene chromosomes.

Data analyses

Data were analyzed for the following three aspects:

Assessment of intermediate time needed for stage-specific development

It was done by counting the number of days required for the developing flies to reach a particular stage of development under our study, *i.e.* third instar larva, pupa or adult from the day (Day 0) on which parental male and female flies were released into each culture vial for mating.

Assessment of the number of representative individuals of pupal and adult stages

It was done by counting the total number of pupae and young adults appeared in each culture vial till 5 days from the initial day of their appearance.

Assessment of chromosomal rearrangements:

It was done by high resolution microscopic (Olympus Microscope, Japan, Model L-200A) examination of the prepared polytene chromosomes in the glass slides obtained from third instar larvae of control and test groups. Photographs of selected chromosomal aberrations were taken at $\times 100$ magnification by a camera (Nikon, Japan, Model- EH-53).

Statistical analyses

Numerical values were expressed as the mean \pm standard deviation (SD). Two tailed t test was performed to determine the deviation, if any, of the test groups from the controls with respect to the number of days required for initial appearances of third instar larvae, pupae and young adults from Day 0. Welch t test was performed to determine the impact, if any, of the salts on the overall population size that included the number of pupae and young adults emerged. Probability (p) values of <0.05 were considered significant.

Results

Assessment of intermediate time needed for stage-specific development

Table 1 shows that the number (mean \pm SD) of days needed for the initial appearances of third instar larvae, pupae and young adults from Day 0 increased dose dependently, compared to controls, owing to the exposure of flies to increasing concentrations of NaNO_2 and KNO_2 , i.e. 10 mM, 20 mM and 30 mM. The delays in appearances of specific developmental stages were more extensive in cultures exposed to 20 mM and 30 mM concentrations of NaNO_2 and KNO_2 . Two-tailed t -test was performed with every concentration of NaNO_2 and KNO_2 with control flies. The p value of each concentration of NaNO_2 and KNO_2 with regard to interval time between the initial days of appearance of various developmental stages and Day 0 is given in Figure 1.

From Figure 1, it is evident that the time interval at only 30 mM NaNO_2 concentration gives the p -value ≤ 0.05 , which is under accepted level of statistical significance at 95% confidence level and 4 degrees of freedom. All other p -values with respect to other concentrations

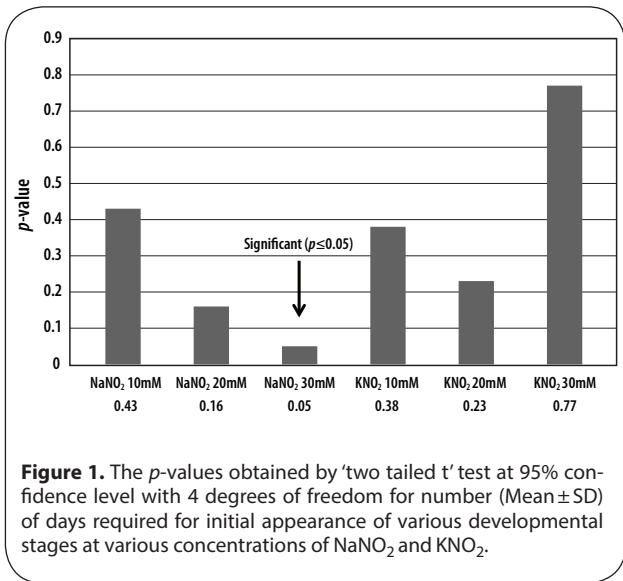


Figure 1. The p -values obtained by 'two tailed t ' test at 95% confidence level with 4 degrees of freedom for number (Mean \pm SD) of days required for initial appearance of various developmental stages at various concentrations of NaNO_2 and KNO_2 .

of either NaNO_2 or KNO_2 were found to be statistically insignificant ($p \geq 0.05$). Exposure to 40 mM concentration of either NaNO_2 or KNO_2 proved to be lethal as no larval development was observed at this concentration (data not shown in Table 1).

Treatment of NaNO_3 and KNO_3 exhibited no statistically significant difference from control with regard to the number (mean \pm SD) of days needed from Day 0 for the initial appearance of third instar larvae, pupae and young adults. Even the exposure of flies to NaNO_3 and KNO_3 at 40 mM concentration yielded no effect on the time needed for the conversions of successive stages of development of the flies compared to control, as shown in Table 2.

Table 1. Time course of developmental stages of *Drosophila melanogaster* exposed to NaNO_2 and KNO_2 .

Developmental Stages	Control	NaNO_2 10 mM	NaNO_2 20 mM	NaNO_2 30 mM	KNO_2 10 mM	KNO_2 20 mM	KNO_2 30 mM
3 rd Instar Larva	3.3 \pm 0.5	5.4 \pm 0.5	7.9 \pm 0.7	12 \pm 1.0	5.8 \pm 1.1	7 \pm 0	10.2 \pm 1.1
Pupa	5.3 \pm 0.5	8.2 \pm 1.3	11.4 \pm 0.8	15.7 \pm 1.1	8.2 \pm 0.9	10 \pm 1.2	13.8 \pm 1.8
Adult	10.1 \pm 0.7	13.2 \pm 0.4	18.3 \pm 2.2	23.7 \pm 0.8	13.8 \pm 1.1	16.2 \pm 1.5	20.5 \pm 1.0

Number (Mean \pm SD) of days required for initial appearances of the particular stages of development of *Drosophila melanogaster* from the day (Day 0) on which adult flies were released into the control and test culture vials with varying concentrations of NaNO_2 and KNO_2

Table 2. Time course of developmental stages of *Drosophila melanogaster* exposed to NaNO_3 and KNO_3 .

Developmental Stages	Control	NaNO_3 10 mM	NaNO_3 20 mM	NaNO_3 30 mM	NaNO_3 40 mM	KNO_3 10 mM	KNO_3 20 mM	KNO_3 30 mM	KNO_3 40 mM
3 rd Instar	3.3 \pm 0.5	3.3 \pm 0.5	3.1 \pm 0.4	3.3 \pm 0.5	3.3 \pm 0.5	3.3 \pm 0.5	3.3 \pm 0.5	3.3 \pm 0.5	3.3 \pm 0.5
Pupa	5.3 \pm 0.5	5.3 \pm 0.5	5.3 \pm 0.5	5.7 \pm 0.8	5.3 \pm 0.5	5.3 \pm 0.5	5.3 \pm 0.5	5.2 \pm 0.4	5.5 \pm 0.5
Adult	10.1 \pm 0.7	10.7 \pm 0.8	10.5 \pm 0.5	10.5 \pm 0.8	10.8 \pm 1.0	10.7 \pm 1.0	11.2 \pm 0.8	10.6 \pm 0.5	10.7 \pm 0.5

Number (Mean \pm SD) of days required for initial appearances of the particular stages of development of *Drosophila melanogaster* from the day (Day 0) on which adult flies were released into the control and test culture vials with varying concentrations of NaNO_3 and KNO_3

Table 3. Population data of *Drosophila melanogaster* exposed to NaNO₂ and KNO₂.

Developmental stages	Control	NaNO ₂ 10 mM	NaNO ₂ 20 mM	NaNO ₂ 30 mM	KNO ₂ 10 mM	KNO ₂ 20 mM	KNO ₂ 30 mM
Pupa	56±6	49±6	28±7	9±3	58±8	37±10	25±14
Adult	59±11	48±7	21±7	1±1	50±6	29±12	10±2

Number (Mean ± SD) of pupae and young adults appeared up to 5 days from their initial days of appearance in control and test culture vials with varying concentrations of NaNO₂ and KNO₂

Table 4. Population data of *Drosophila melanogaster* exposed to NaNO₃ and KNO₃.

Developmental stages	Control	NaNO ₃ 10 mM	NaNO ₃ 20 mM	NaNO ₃ 30 mM	NaNO ₃ 40 mM	KNO ₃ 10 mM	KNO ₃ 20 mM	KNO ₃ 30 mM	KNO ₃ 40 mM
Pupa	56±6	43±5	52±8	48±2	43±10	52±3	51±3	47±4	46±6
Adult	59±11	24±5	19±7	16±5	15±2	47±3	41±6	32±3	32±4

Number (Mean ± SD) of pupae and young adults appeared up to 5 days from their initial days of appearance in control and test culture vials with varying concentrations of NaNO₃ and KNO₃

Assessment of the number of representative individuals of pupal and adult stages:

Examination of the number (mean ± SD) of pupae and adults generated till 5 days from their initial days of appearances, as shown in Table 3, reveals that all the cultures exposed to varying concentrations of NaNO₂ and KNO₂ showed dose dependent reduction in their number with increasing concentration of both of these salts compared to controls. However, the hallmark of inhibition was observed in cultures exposed to 30 mM NaNO₂, where the number of pupae and adults generated were reduced by 84% and 98% respectively in comparison to controls.

Welch *t*-test was performed with every concentration of NaNO₂ and KNO₂ to find out the statistically significant impact, if any, of the salts on the overall population size that included the number of pupae and young adults emerged. The *p* values of each concentration of NaNO₂

and KNO₂ with respect to overall population size (mean ± SD) in controls is given in Figure 2.

From Figure 2, it is evident that only 30 mM NaNO₂ gives a *p*-value ≤ 0.05, which indicates that reductions in the numbers of pupae and adults at this concentration of the salt were statistically significant. Exposure to NaNO₃ and KNO₃ revealed no significant difference on the pupal or adult output in comparison to control. The most interesting observation was that development of flies occurred even on their exposure to 40 mM NaNO₃ and 40 mM KNO₃ (Table 4). This is in contrast to the situation where exposures to both 40 mM NaNO₂ and 40 mM KNO₂ proved to be lethal for the developing flies.

Assessment of chromosomal rearrangements

Polytene chromosomes prepared from the third instar larvae of the flies exposed to only NaNO₂ and KNO₂ at 20 mM and 30 mM concentrations exhibited various chromosomal rearrangements, like deletion looping (Figure 3), inversions (Figure 4), regional shrinking, formation of highly enlarged puffs (Figures 5 and 6), etc. Neither any lesser concentration of these two salts nor any concentration of nitrate salts or any control culture was associated with these types of chromosomal abnormalities in larvae.

Discussion

Drosophila melanogaster has been used in our present study owing to its extensive genetic homology to mammals (Deepa *et al.*, 2012; Pandey & Nichols, 2011). A large number of highly conserved genes with similar biological roles exists both in humans and in *Drosophila* and mutations of some of these genes can affect the comparable systems and organs in both these organisms (Banfi *et al.*, 1997). Nearly 75% of the genes associated with a known human disease have conserved counterparts in this fly (Pandey & Nichols, 2011). Furthermore, *Drosophila melanogaster*

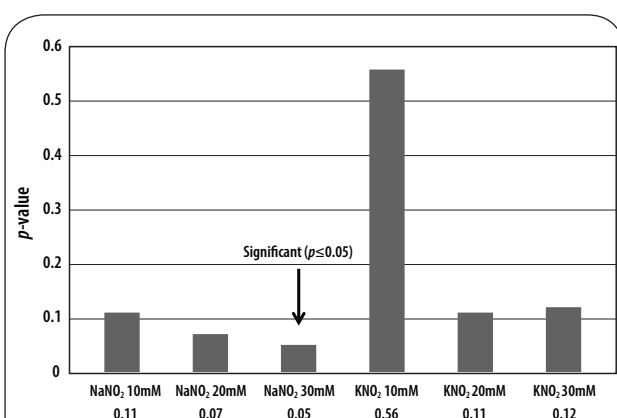


Figure 2. The *p*-values obtained by 'Welch *t*' test at 95% confidence level with 1 degree of freedom for overall population size that included the number (Mean ± SD) of pupae and young adults emerged due to exposure to various concentrations of NaNO₂ and KNO₂.

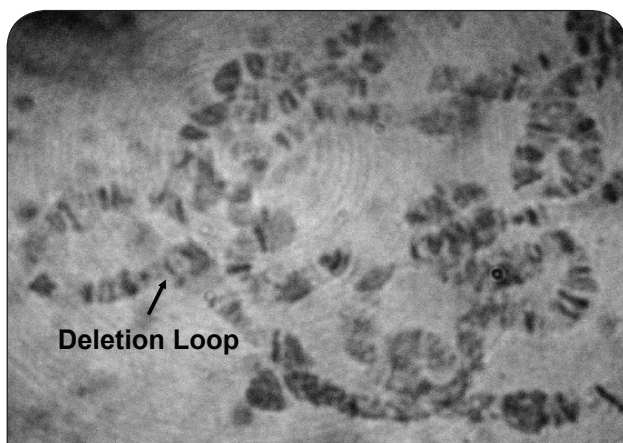


Figure 3. Formation of deletion loop in a polytene chromosome obtained from third instar larva exposed to 30mM NaNO_2 in culture medium.

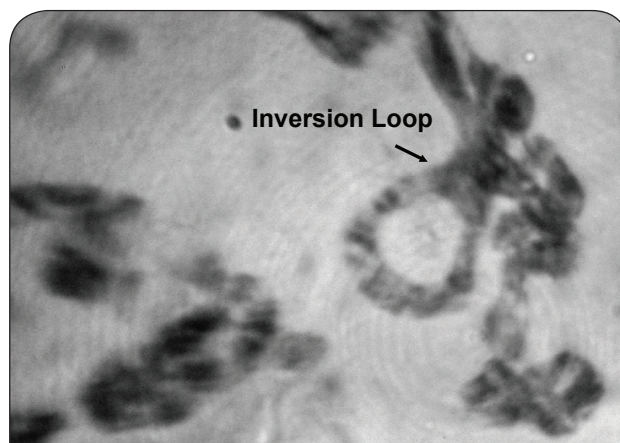


Figure 4. Formation of inversion loop in a polytene chromosome obtained from third instar larva exposed to 30mM NaNO_2 in culture medium.

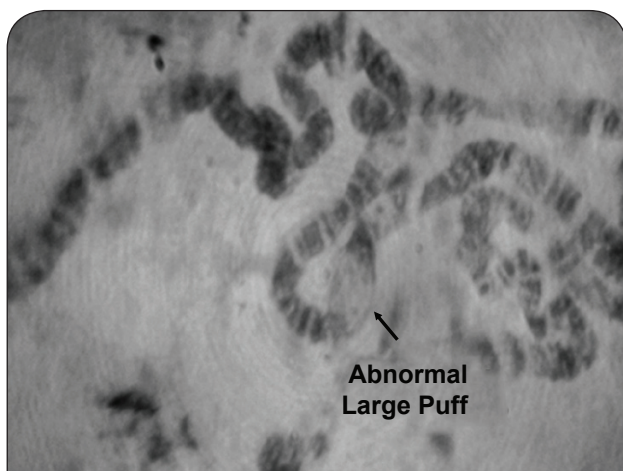


Figure 5. Formation of an abnormally large puff in a polytene chromosome obtained from third instar larva exposed to 30mM NaNO_2 in culture medium.

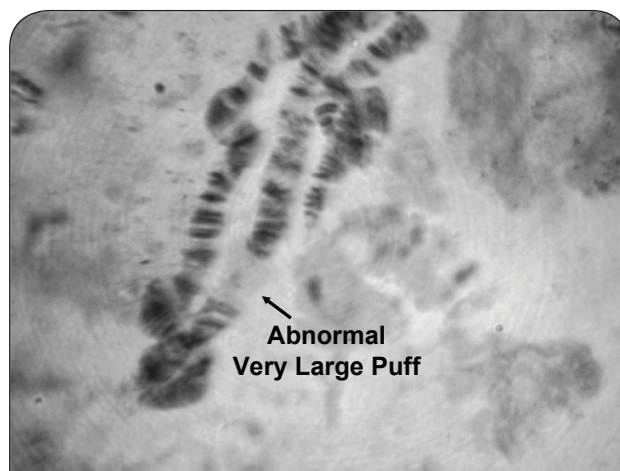


Figure 6. Figure showing formation of an abnormally very large puff in a polytene chromosome obtained from third instar larva exposed to 30mM NaNO_2 in culture medium

has been a very useful model for developmental biology (Sahai-Hernandez *et al.*, 2012) and is getting much application in toxicological studies (Hirsch *et al.*, 2003; Rand & Dao, 2009; Rand *et al.*, 2014). We thus speculate that the adverse effects of exposures of the food additives, which we observed during the development of *Drosophila*, reflect analogous situations that may occur during human development owing to maternal intake of these chemicals and compel us to reconsider the safety issue of their dietary use during human pregnancy. We have chosen sodium and potassium salts in our study as these salts have been used for many years to prevent the growth and toxin production by *Clostridium botulinum*. Human exposure to nitrates and nitrites also occurs through the intake of fruit and vegetables, as well as through drinking water (Hord *et al.*, 2009). In addition, sodium and potassium nitrate are also added to certain cheese for their preventive effects against gas forming bacteria (Topçu *et al.*,

2006; <http://www.foodstandards.gov.au>). Many human studies have revealed that nitrate and nitrite preserved meat intake is associated with various forms of cancer (Linseisen *et al.*, 2006; Aschebrook-Kilfoy *et al.*, 2013). Nitrate and nitrites are precursors of nitroso compounds *in vivo* (Björne, 2004; Suzuki *et al.*, 2003; Suzuki *et al.*, 2005; Erkekoglu and Baydar, 2010; Huber *et al.*, 2013) that induce the formation micronucleus, representing a biomarker for human cancer (Hebels *et al.*, 2011). Thus with respect to the reported potential health hazards of nitrite and nitrate salts, we have considered the common food preservatives like sodium and potassium salts of nitrate and nitrites for our experiments.

In our present study, out of the four food additives, only NaNO_2 and KNO_2 completely arrested the development of flies at 40 mM concentration in culture medium. However, NaNO_3 and KNO_3 at this particular concentration exerted no significant effect on the normal

time course of each developmental phase, nor in the number of emerging pupae and young adult flies. Thus it appears that dietary consumption of NaNO_3 and KNO_3 during development is not harmful to flies, at least in the concentration range of these food additives used in our experiments. In contrast, both NaNO_2 and KNO_2 seem to be lethal at 40 mM concentration as no development of the flies occurred at this concentration. Among the other concentrations of the salts examined, exposure of flies to only 30 mM NaNO_2 resulted in significant increase in time required for conversion of successive developmental stages of the flies as well as in significant reduction of the number of pupae and young adults emerged compared to controls. The decline in the number of emerging individuals of the two stages of development was substantial as only 16% pupae and as low as 2% adults finally emerged due to the exposure of the flies at this concentration compared to the control population. Dietary inclusion either of NaNO_2 below this concentration or of KNO_2 up to 30 mM concentration resulted in no statistically significant correlation on the overall developmental outcome of the flies.

In one study (Sarikaya & Cakir, 2005) researchers in a somatic mutation and recombination test (SMART) exposed 3-day-old *Drosophila* larvae to NaNO_2 , KNO_2 , NaNO_3 and KNO_3 under a concentration range of 25 mM to 100 mM to explore their genotoxic effects, which became phenotypically evident during adulthood of the flies. In this study, however, larvae attained adulthood even on their exposure to 100 mM concentration of all the salts mentioned above. This finding is in contrast to our result that showed complete paucity of development of the flies at 40 mM concentrations of NaNO_2 and KNO_2 . We speculate that the reason for the discrepancies in the experimental outcomes of the two groups may be due to the fact that in our case the flies received toxic exposures of these salts from their very early developmental stage, *i.e.* from their embryonic stage, when they were probably more vulnerable to the toxic effects of the salts compared to 3-day-old larvae used by the group mentioned above. However, one consensus of the experimental findings of the two groups is that NaNO_2 was proved to be the most toxic among the four different salts examined. NaNO_2 exposure was reported to be associated with DNA damage in experimental rodents (Ohsawa *et al.*, 2003). Such DNA damaging activities of NaNO_2 may be due to the activities of oxygen radicals as nitrites in the acidic environment of the mammalian stomach react with amines and amides to form nitrosamines (Erkekoglu and Baydar, 2010; Huber *et al.*, 2013) that can produce ROS, powerful DNA damaging agents (Bandyopadhyay *et al.*, 1999; Sharma *et al.*, 2012) in the cells. We hypothesize that ROS may be generated in the acidic environment of the gut of *Drosophila* larvae (Dubreuil *et al.*, 2001; Dubreuil, 2004; Overend *et al.*, 2016) fed with NaNO_2 in our experiment, leading to developmental inhibitions by damaging the genetic material. Since multiple chromosomal structural abnormalities can be detected in the polytene chromosomes of *Drosophila* and other dipterans in response to exposure to

chemicals and toxin (Michailova *et al.* 2001; Singh *et al.*, 2012; Turgay *et al.*, 2005; Uysal, 1997), we examined these giant chromosomes of the third instar larvae of the flies, exposed to varying doses of NaNO_2 , NaNO_3 , KNO_2 and KNO_3 , to ascertain whether the developmental inhibition of the flies was accompanied with changes in the genetic material or not. Examinations of polytene chromosomes of the third instar larvae revealed the presence of various forms of chromosomal rearrangements like inversions, deletion loopings, regional shrinking and highly enlarged large puffs in larvae exposed to 20 mM and 30 mM concentrations of NaNO_2 and KNO_2 . We found that both concentrations of these two salts negatively affected the development of flies, although statistically significant developmental inhibition in culture was shown by exposure to 30 mM NaNO_2 only. The occurrence of chromosomal lesions at NaNO_2 , KNO_2 concentrations those did not result in any significant difference in the time course of development and in the number of emerging individuals of the flies in comparison to controls may be due to the fact that ROS generation at these concentrations was below the threshold level to make substantial DNA damage for developmental inhibitions to occur. ROS can produce many kinds of DNA lesions, like deletion, frame-shift, modification of bases, nucleotide removal, strand breakage, DNA protein cross links, chromosomal rearrangements, causing mutations and cellular death (Bandyopadhyay *et al.*, 1999; Sharma *et al.*, 2012; Valko *et al.*, 2007; Valko, 2004). Chromosomal rearrangements like deletions, inversions are caused by breakage of DNA double helices at two different locations followed by the rejoining of their broken ends that generate different arrangements of the order of the genes from the original one (Griffiths *et al.*, 1999). These rearrangements may distort the integrity between a gene and its regulatory region close to the breakpoint leading to changes in the expression of the genes or disruption of the gene (Muñoz & Sankoff, 2012; Raeside, 2014). Damage in the DNA can also alter the structure of proteins encoded by the genes that can lead to functional anomalies or complete loss of function of the proteins (Sharma *et al.*, 2012). A deletion in a chromosome is identified as a loop if the individual also possesses a normal homolog that during meiosis bulges out due to the failure of the normal homolog to pair at the deleted region on the other homologous chromosome. The resulting reduction of some genes by half may lead to haploinsufficiency, *i.e.* expression of a single wild type allele unable to produce normal phenotype (Griffiths *et al.*, 1999; Vijg & Suh, 2007; Hyde & Carrow, 2010). Furthermore, expression of a single copy of some genes may cause insufficient expression of the remaining genes leading to genetic imbalance (Hyde & Carrow, 2010). In inversion, the region between the breaks on a chromosome undergoes rotation of 180 degrees, followed by the reinsertion of the reversed piece back into the chromosome. If the break occurs within a gene of essential function, lethality may occur (Vijg & Suh, 2007). In addition, inversions alter gene expression by separating regulatory elements from the coding sequences or by

providing the coding sequence a new regulatory region (Puig *et al.*, 2015). “Puffs” are localized expansions of the polytene chromosome structures, which are the sites of synthesis of RNA (Korochkin, 1981). However, novel sets of puffs are induced in the polytene chromosomes of *Drosophila* as stress response following exposure to temperature and chemicals (Singh *et al.*, 2012). Indeed many chemicals such as formamide, acetamide and nicotinamide, benzamide, colchicine, thiamphenicol, paracetamol, *etc.* induce a high rate of transcription at a puff which is induced normally by heat shock (Tapadia & Lakhotia, 1997). In our present study, exposures of higher concentrations of NaNO₂ were accompanied with the appearance of unusually large puffs in *Drosophila* larvae, which we suppose resulted due to chemical stresses induced by this salt. Considering the above discussed alterations in gene expressions, which may occur due to various chromosomal rearrangements, we speculate that exposure of flies in our case to 30 mM NaNO₂ during development possibly altered the embryonic and larval gene expressions drastically by chromosomal rearrangements, as shown by our results, leading to significant developmental inhibitions of the flies. Probably exposure of flies to 40 mM NaNO₂ and 40 mM KNO₂ might cause DNA damage to such an extent that the embryonic gene expression machinery collapsed abruptly, leading to complete developmental inhibition.

Our present study revealed that nitrate salts did not result in any negative influence in the development of the flies. The probable reasons for such observation is that nitrates in humans are quickly excreted in urine (Gilchrist *et al.*, 2011) and are relatively inert substances until they are converted into nitrite (Jones *et al.*, 2015). It has been reported that approximately only 5% of ingested nitrate is converted into nitrite in the saliva in human subjects (National Academy Press, Washington DC). Thus the contribution of dietary nitrates for nitrosamine compound formation seems to be far lower than the direct consumption of nitrites. No data are available so far regarding the salivary conversion of nitrate into nitrite in the larvae of *Drosophila* or other insects. We speculate that even if such a system exists, nitrates probably play a small role as precursors of nitroso compounds in the larval gut. The reason for such a speculation is based on the fact that nephrocytes of the *Drosophila* excretory system have remarkable anatomical, molecular and functional similarities with vertebrate glomerular podocytes that act as a barrier during the ultrafiltration of blood for making urine. The similarities between these two cells provide evidence to suggest that they are evolutionary related. Nephrocytes have been designated as ‘storage kidneys’ because of their function to regulate hemolymph composition. They are derived from the embryonic mesoderm and many persist through metamorphosis into adult life of *Drosophila* (Weavers *et al.*, 2009; Denholm & Skaer, 2009). There is thus the possibility that nitrate may be readily excreted by nephrocytes of *Drosophila* larvae making the salt less responsible for conversion into nitrite *in vivo* and thus hindering the developmental process.

We infer that owing to extensive genetic and functional homology between *Drosophila* and human, detrimental effects shown by NaNO₂ and KNO₂ during the development of this fly in our study advocate cautious dietary use of these food additives to avoid possible fetal losses or birth defects in humans.

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Conflicting Interest

The authors declare that there are no conflicts of interest.

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