EVALUATION OF GENOTOXICITY OF THREE FOOD PRESERVATIVES IN DROSOPHILA MELANOGASTER USING SMART AND COMET ASSAYS

Ingy El-Hefny1*, Walaa Hozayan2,3, Neima AlSemeny4, Wesam Basal5, Amr Ahmed2, and Ayman Diab1

Address(es):
1October University for Modern Sciences and Arts, (MSA), Faculty of Biotechnology, Wahat Road, 6th of October City, Egypt, Tel: +201276243198 / +201225125141.
2Beni-Suef University, Faculty of Postgraduate Studies for Advanced Sciences, Department of Biotechnology and Life Sciences, Beni-Suef, Egypt.
3Beni-Suef University, Faculty of Science, Biochemistry Division, Chemistry Department, Beni-Suef, Egypt.
4Ain Shams University, Faculty of Agriculture., Department of Genetics, Shubra el-Kheima, Egypt.

*Corresponding author: ingyelhefny@hotmail.com
doi: 10.1541/jmbfs.2020.10.1.38-41

ARTICLE INFO
Received 28. 11. 2018
Revised 2. 3. 2020
Accepted 4. 3. 2020
Published 1. 8. 2020

ABSTRACT
The continuously growing food and beverage industry relies on food additives as a main component in their products. Such increased reliance on processed food, lead to neglectance of the harmful effects of the food additives on human health; among these are hypersensitivity, allergic reactions, genotoxicity, mutagenicity and more. This study investigates genotoxic effects of three food preservatives commonly consumed in daily meals; sodium sulphite, boric acid, and benzoic acid using the somatic mutation and recombination test (SMART) and comet assay in Drosophila melanogaster system. All of the tested compounds showed significantly high levels of tumor induction and frequency compared to a negative control in SMART assay. They also showed high amount of DNA damage in the comet assay indicating their high potential of being genotoxic materials.

INTRODUCTION
Food additives are substances frequently added to food to enhance the quality of the final product in several aspects; extending life time by retarding or inhibiting growth of microorganisms, colouring, sweetening, flavouring and thickening (Rekha and Dharman, 2011). For a long time, no observed adverse effects have been proven on the basis of toxicological studies. However, several studies had proven that the consumption of some additives in processed food might have increased the risk of human cancer despite the respected legal limits of these additives by the manufactures. The carcinogenic risk of food additives can be attributed to various factors; interaction of additives with some food ingredients, chemical formula of food additives might be changed during food processing to a carcinogenic formula, a negative synergistic effects when combined with other additives, unsuitable storage conditions, and unknown carcinogenic by-products occurring during the food processing (Gálisoy et al., 2015). Sodium sulphite, a preservative used to stop the browning and further ripening of fruits, was found to induce inhibition of DNA synthesis in Vicia faba root, bridges in anaphase and chromatin erosion of interphase nuclei (Njagi and Gopalan, 1982).

According to (Olorunfemi et al., 2012), tests carried out at cytogenetic anomalies Allium cepa reveals a decrease of the mitotic index caused by treatments applied. Mitosis analysis indicates the development of a number of structural chromosomal aberrations and interphases aberrations identified in the different stages of mitosis, the process of cell division being significantly affected.

Benzoic acid, a commonly used preservative as antimicrobial substance in many food products, was found to cause a weak positive increase in chromosomal aberration test in CHO cells (Ishidate et al., 1984). It also induced somatic mutations in Drosophila SMART test (Sarikaya and Solak, 2003) and increased the chromosomal aberration, sister chromatid exchange, and micronucleus frequency in human peripheral lymphocytes (Yilmaz et al., 2009; Al-Tai et al., 2014). Borax, a salt of boric acid, had an inhibitory effect on HepG2 cell growth and induced apoptosis in a concentration-dependent manner (Wei et al., 2016). Among the most accepted tests that are applied to assess the carcinogenic potential of a given substance is the Somatic Mutations and Recombination Test (SMART) carried out in Drosophila melanogaster (Demir et al., 2013). This assay uses tumor suppressor gene warts which is a homolog to the mammalian tumor suppressor gene p53 (Nepomuceno, 2015; Vasconcelos et al., 2017). The evolutionary conservation of tumor suppressor genes among Drosophila and mammals has prompted studies of tumor induction in Drosophila, such studies has contributed to the understanding of cancer in human (Potter et al., 2000; Eenek et al., 2002). Homozygosity loss of the warts gene induced by mitotic recombination in somatic cells leads to the formation of greatly overgrown cell clones that can be easily detected as tumors on fly body (Justice et al., 1995). SMART is a rapid, very sensitive to different classes of agents and inexpensive assay which is able to evaluate the carcinogenic activity of single compounds as well as complex mixtures. It also allows various protocols for the application of the test materials as single, combined or sequential treatments of the larvae. Factors capable of Inducing tumors in Drosophila instead of marker clones might directly adverse the risk of these factors for inducing cancer in humans (Sidorov et al., 2001). In flies heterozygous for the wts gene, the genetic events that can lead to the tumor appearance and hence can be detected by SMART include; gene mutations in the wts gene, multilocus-deletions (partial), chromosomal loss and somatic recombination collectively referred to as loss of heterozygosity (Eeken et al., 2002).

Comet assay (also called, single cell gel electrophoresis, SCGE) is an assay used over the last few decades to detect any prospective damage for DNA after certain treatments. The assay is able to detect DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilized nuclear chromatin. This assay is one of the most widely accepted tests for detection of DNA damage as it offers several advantages over the other tests, these include: (1) damage to the DNA in individual cells is measured; (2) only small number of cells are needed to carry out the assay (<10,000); (3) the assay can be performed on virtually any eukaryotic cell type; (4) and it is faster and more sensitive than the alkaline elution method for detecting DNA damage DNA (Singh et al., 1998). Comet assay is a useful tool for the evaluation of local genotoxicity, particularly organs or cell types, which can hardly be evaluated with other standard tests (Brendler et al., 2005). Thist assay has become the prime choice in the assessment of DNA

Keywords: mutagenic, genotoxic, comet, Drosophila, food additives
damage and genotoxicity testing considering that it is an easy to perform, short time and low cost test that requires small numbers of cells/sample. Moreover, it is sensitive for detecting low levels of DNA damage. Alkaline comet assay (pH 13), the most commonly used version, is able to detect all possible kinds of DNA damage (Tice et al., 2000). In recent years, the comet assay has been adapted to use in vivo in Drosophila (Mukhopadhyay et al., 2004; Shukla et al., 2011), to combine its advantages with those well-established of this fly. The objective of this study was to evaluate the genotoxic and carcinogenic effects of three food additives; sodium sulphite, boric acid, and benzoic acid using SMART and comet assays in Drosophila.

MATERIAL AND METHODS

Somatic Mutation and Recombination Test (SMART) in D. melanogaster

Drosophila melanogaster strains

Two different Drosophila strains were used in this study; wild type strain and a strain that carries wtsMT+, a lethal warts allele balanced on TM3, characterized by multiple inversions and marked by the dominant mutation stubble according to Eiken et al. (2002) and fly base (2006). The genetic structure of this strain is, st p in r wtsMT4-U TM3 Sb, which was abbreviated wts/TM3. Details about the various markers and the balancer chromosome can be found in Lindsey and Zimm (1992).

Crosses and treatments

The wts/TM3 females were crossed to wild type males resulting in two genotypes offspring, wts/+ and TM3, Sb wts-+. After 2 days, the parental flies were removed and 56-68 hours old larvae were washed with 20% glycerol, then collected using a fine mesh sieve and transferred to five different vials representing the five test groups. For food additives treated groups (Sodium sulphite, Boric acid, and Benzoic acid); the flies were transferred to a standard Drosophila medium to which a 100 mM of each food additive powder was added and properly dissolved at 50°C. The larvae were submitted to chronic treatment for approximately 48 h, then they were transferred to standard Drosophila medium. The positive control group was transferred to a vial where 20 µg/ml of an appropriate Mitomycin C (MMC) solution was mixed with a standard Drosophila medium, kept for 24 hours, then they were transferred to standard Drosophila medium. Negative control group was directly transferred to a standard Drosophila medium. Afterwards, larvae of all groups were left to feed on the medium until completion of their development when they leave the medium and pupate. All Drosophila stocks and crosses were maintained at 25°C. Only adult flies, without the chromosome balancer (TM3, Sb) with no truncated bristles were analysed.

Scoring of Warts:

After metamorphosis, the adult flies were transferred to flasks containing 70% ethanol. Flies were analysed for tumour presence using a Leica stereomicroscope used at a standard magnification of 25 X and an entomological tweezers. Only tumors that were large enough to be unequivocally classified are recorded (Eeken et al., 2002).

Statistical Analysis

The tumor frequency was calculated as the number of tumors/number of wts+/+

| Treatments          | Total No. of Fly Scored | No. of Fly Scored with Tumor | No. of Tumor Scored | Tumor Induction | Frequency (No. of Tumors/Fly ± S.D.) |
|---------------------|-------------------------|------------------------------|---------------------|-----------------|-------------------------------------|
| Negative Control    | 950                     | 61                           | 69                  | 1.1             | 0.07±0.06                           |
| MMC 20µg/ml         | 765                     | 465                          | 1016                | 2.18            | 1.33±0.45                           |
| Sodium Sulphite     | 682                     | 305                          | 474                 | 1.55            | 0.69±0.11*                          |
| Boric Acid 100mM    | 601                     | 291                          | 486                 | 1.67            | 0.8±0.14**                          |
| Benzoic Acid 100mM  | 800                     | 490                          | 590                 | 1.2             | 0.73±0.18*                          |

* and ** significant, highly significant difference from the negative control at P<0.05 using Mann, Whitney and Wilcoxon nonparametric U test. Frequency (No. of Tumor/Fly) = Number of tumors/Total number of tested flies. Tumor induction = Number of tumors/Number of tumor flies.
DNA damage was assessed in adults of the homogenic *Drosophila* strain w*1118* emerged from 2nd larval instar exposed to three tested compounds. According to DNA damage parameters; tailed%, untailed %, tails length, tail DNA% and tail moment, all of the three tested salts caused significant DNA damage Table(2). Moreover, an increase in tail length was observed in sodium sulphite, boric acid and benzoic acid treated groups as compared to control group (Fig. 2), which is a clear indication on DNA degradation and strand breaks. Migration length is considered to be directly related to fragment size and proportional to the level of single stranded breaks and alkali-labile sites (Tie et al., 2000).

Benzoic Acid had significantly higher deleterious effect on DNA of *D. melanogaster* (8.5±0.58 µm compared with control 1.29±0.10) than Sodium Sulphite (6.90± 0.50 µm tails length) and Boric Acid (5. 34±0.19 µm tails length). Tail DNA percentage was 1.45% in control, 7.11% in Benzoic Acid treatment, 5% in Sodium Sulphite treatment and 4.15% in Boric Acid as recorded in Table (2).

Table 2 Detection of DNA damage by the comet assay, assessed as tail moment (TM) in whole body cells of white eye adult *Drosophila* treated with the sodium sulphite, boric acid and benzoic acid.

| Group          | Tailed % | Untailed % | Tails length µm | Tail DNA% | Tail moment |
|----------------|----------|------------|-----------------|-----------|-------------|
| Control       | 2        | 98         | 1.29±0.10       | 1.45      | 1.83        |
| P.N.F.N       | 17       | 83         | 6.90± 0.50*     | 5         | 34.56       |
| Sodium Sulphite | 10       | 90         | 5. 34±0.19*     | 4.15      | 16.12       |
| Benzoic Acid | 25       | 75         | 8.5±0.58**      | 7.11      | 60.15       |

*and ** significant, highly significant difference from the negative control at P<0.05

This study evaluated the potential genotoxicity and carcinogenicity of three food additives commonly used in food industry using SMART test on *Drosophila melanogaster* system and comet assay. The obtained results clearly reveals the genotoxic potential of the tested food preservatives. Formation of tumors in *Drosophila* SMART assay is a strong indication for Loss of heterozygosity (LOH) in somatic cells. The mechanism might involve mutation, chromosome loss or somatic recombination. Further studies will be carried out to investigate the mechanism of LOH. The use of alternative small organisms as models in toxicology has grown tremendously in the last decade. Drosophila has always been a premier model for both developmental biologists and geneticists, however, several recent toxicology studies have used this organism. Currently *Drosophila* is being used in studies of a number of priority environmental contaminants and toxicants (rand et al., 2015). The striking resemblance between human and *Drosophila* genes and the presence of numerous highly conserved genes and pathways controlling development of stress response across these two divergent species suggests that the genotoxic potential of the tested might have the same effect on human that cannot be ignored.

Benzoic Acid was found to be concentration dependent, while that caused by borax was in both concentrations. They were also found to stimulate a significant decrease in mitotic index and increase the frequency of chromosomal aberration in human lymphocytes (El-Hefy et al., 2014) The impact of sodium metabisulphite and boric acid on somatic cells of *Vicia faba* L. was studied by (Pandey and Upadhyay, 2007). They found a significant decrease in mitotic index in human lymphocytes (Meng and Zang, 1992; Rencuzogullari et al., 2001). Results obtained from comet assay have backed up the genotoxic potential of these food additives considering the significant amount of DNA damage in cells treated by the tested compounds compared to negative control. The mechanism of boric acid and borax genotoxicity was assessed in zebrafish Denio rerio after 24, 48, 72 and 96-hours acute exposure level to 1, 4, 16, 64 mg/l of each of the tested compounds in semi-static bioassay experiment. Peripheral erythrocytes were drawn from caudal vein and subjected to Comet assay to assess genotoxicity. The amount of DNA damage caused by boric acid was found to be concentration dependent, while that caused by borax was in both concentration and time dependent manner (Gülsoy et al., 2015). A significant increase in mean tail intensity and mean tail length were observed by Meng et al. (2014) in human lymphocytes exposed to 50-500 µg/ml concentrations of benzoic acid. In another study, when human male germ cells were exposed to different concentrations 50, 100, 200 and 500 µg/ml of benzoic acid, the results indicated that the concentrations starting from 200 µg/ml showed a significant increase in tail DNA%, tail length and tail moment (Pandir 2016). Meng et al. (2004) investigated the in vivo effects of sodium sulphite and sodium bisulfite on various organs (brain, lung, heart, liver, stomach, spleen, thymus, bone marrow and kidney) of male mice. They found significant increases in DNA damage providing further evidence for a systemic toxic activity of sulfur dioxide derivatives.

![Figure 1](image1.png)

**Figure 1** Diagram represents the tumor induction of spontaneous and induced warts Epithelial tumors in +/- wts flies after treatments with Mitomycin C (MMC), Sodium sulphite, Boric acid, and Benzoic acid.

![Figure 2](image2.png)

**Figure 2** DNA damage in adult *Drosophila* whole body cells, Comet images of DNA strand breaks of control (A), (B) and (C) and (D) DNA damaged after exposure to sodium sulphite, boric acid and benzoic acid respectively.
CONCLUSION
It can be safely concluded from the present and previous work that all of the tested compounds has a noticeable genotoxic potential that cannot be ignored while using such chemicals in the food industry. Further thorough investigations are recommended before continuing using these substances in food and cosmetics as additives.

REFERENCES
AI-Tai, E.M.F. (2014). Protective Effect of pomegranate molasses (PM) Against Genotoxicity Induced by Benzoic acid (E-210) in human lymphocytes in vitro. Nature and Science, 12(11),13-16. http://www.sciencenet.net/nature3
Barranco, W.T., Eckert, C.D., (2004). Boric acid inhibits human prostate cancer cell proliferation. Cancer Lett., 216(1), 21-29. https://doi.org/10.1016/j.canlet.2004.06.001
Brendler-Schwaab, S., Andreas, H., Stefan, P. & Speit, G. (2005). The in vivo comet assay: use and status in genotoxicity testing . Mutagenesis, 20(4), 245–254, https://doi.org/10.1093/mutage/ged033
Demir, E., Kocaölçü, S. & Kaya, B. (2008). Genotoxicity testing of four benzyl derivatives in the Drosophila wing spot test. Food Chem. Toxicol., 46(3), 1034-41. https://doi.org/10.1016/j.fct.2007.10.035
Demir, E., Kaya, B., Marcos, R., Kocaölçü Cenkcí, S., & Cetin, H. (2013). Investigation of the genotoxic and antigenotoxic properties of essential oils obtained from two Origanum species by Drosophila wing SMART assay. Turkish Journal of Biology, 37(2), 129 – 138. https://doi.org/10.3906/biy-1209-64
Eeken, C.J., Klink, L., Bert, L., Veen, V. Pastink, A., & Ferro, W. (2002). Induction of Epithelial Tumors in Drosophila melanogaster Heterozygous for the Tumor Suppressor Gene wts. Environ. Mol. Mutagenesis, 40(4), 277-282. https://doi.org/10.1002/em.10119
FlyBase: anatomical data, images and queries. (2006). Nucleic Acids Res., 34(1), 484–488. https://doi.org/10.1093/nar/gkl468
Gilsoy, N., Yavas, C. & Mutlu, 0. (2015). Genotoxic effects of boric acid and boraxin in Zebra fish, Danio rerio using alkaline comet assay. EXCLI J., 14, 890-899. http://dx.doi.org/10.17179/excli2015-404
Ishidate, J. R.M., Sofuni, T., Yoshikawa, K., Hayashi, M., Nohmi, T., Matsunaga, A. (1984). Primary mutagenicity screening of food additives currently used in Japan. Food Chem. Toxicol., 22(8), 623-636. https://doi.org/10.1016/0278-6915(84)90271-0
Justice, R.W., Zilian, O., Woods, D.F., Noll M, et al. (1995). Boric acid inhibits human prostate cancer cell proliferation. Mutation Res., 277(1-2), 181–191. http://dx.doi.org/10.1016/0027-5131(95)00027-7
Sykriotis, G.P., Bohmann, D. (2010). Stress-Activated Cap‘n‘collar Transcription Factors in Aging and Human Disease. Sci, Signaling, 3(112), re3. https://doi.org/10.1126/sci signal.3112re3
Singh, N.P., McCoy, M.T., Tice, R.R., & Schneider, E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual Cells. Exp. Cell Res., 175(1), 184–191. http://dx.doi.org/10.1016/0014-4827(88)90265-0
Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., et al. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicity testing. Environmental and Molecular Mutagenesis, 33(3), 206-221. https://doi.org/10.1002/smc.1098-2280(200003)3:2<206::aid-em8>3.0.co;2-6
Vasconcelos, M.A., Orsolin, P.C., Silva-Oliveira, R.G., Nepomuceno, J.C., & Spanò, M.A. (2017). Assessment of the carcinogenic potential of high intense-sweeteners through the test for detection of epithelial tumor clones (warts) in Drosophila melanogaster. Food Chem. Toxicol., 101, 1-7. https://doi.org/10.1016/j.fct.2016.12.028
Wei, Y., Yuan, F.J., Zhou, W.B., Wu, L., Chen, L., Wang, J.J., & Zhang, Y.S. (2016). Borax-induced apoptosis in HepG2 cells involves p53, Bcl-2, and Bax. Genet. Mol. Res., 15(2). https://doi.org/10.4238/gmr.15028300
Yilmaz, S., Fatma, U., & Deniz, Y. (2009). The in vitro genotoxicity of benzoic acid in human peripheral blood lymphocytes. Cytotechnology, 60(1-3), 55–61. https://doi.org/10.1007/s10616-009-9214-z
Yilmaz, S., Unal, F., Yüzbasoğlu, D. & Celenk, M. (2014). DNA damage in human lymphocytes exposed to four food additives in vitro. Toxicol. Ind. Health, 30 (10), 926-37. https://doi.org/10.1177/0260201213486132
Pandir, D. (2015). DNA damage in human germ cell exposed to the some food additives in vitro. Cytotechnology. 68(4),725–733. http://dx.doi.org/10.1007%2Fs10616-014-9824-v
Patel, D. & Ramani, K. (2017). In vitro determination of genotoxic effects of sodium benzoate preservative on human peripheral blood lymphocytes. Int. J. Res. Biosci., 6(3), 20-26. [IRRBs]
Potter, C.J., Turenchalk, G.S., & Xu, T. (2000). Drosophila in cancer research. Trends Genet., 16(1), 33–39. http://dx.doi.org/10.1016/S0168-9525(00)90178-8
Rand, M.D., Montgomery, L. S., Prince, L., & Vorobjekina, D. (2015). Developmental Toxicity Assays Using the Drosophila Model Cell. Curr. Protoc. Toxicol., 59(1), 1.12.1–1.12. http://dx.doi.org/10.1002/0471140856.tox11225
Rekha, K. & Dharman (2011). Mitotic aberrations induced by sodium benzoate: A food additive in Allium cepa L. Plant Archives, 11(2), 945–947. [Research gate]
Renczogullari,E.,Kayraldiz,A.,Ülah,B.,Ckmak,T.,&Topakta,O.(2001). The cytogenetic effects of sodium metabisulfite, a food preservative in root tip cells of Allium cepa L. Turk. J. Biol., 25(4), 361-370. [Semanticscholar]
Sarikaya, R. & Solak, K. (2003). Genotoxicity of Benzoid Asit Studued in the Drosophila melanogaster Somatic Mutation and Recombination Test (SMART). Gazi Eğitim Fakültesi, Dergisi, 23(3), 19-32. [Semanticscholar]
Shukla, A.K., Prayag, P., & Chowdhuri, D.K. (2011). A modified alkaline Comet assay for in vivo detection of oxidative DNA damage in Drosophila melanogaster. Mutatation Res., 726(2), 222-226. https://doi.org/10.1016/j.mrerg.2011.09.017
Sidrov, R.A., Ugunuven, E.G., Khovanova, E.M., & Belitsky, G.A., (2001). Induction of tumor clones in D. melanogaster v(1)wts heterozygotes with chemical carcinogens. Mutation Res., 498(1-2),181–191. http://dx.doi.org/10.1016/S1383-7581(01)00277-7
Sawada, H., et al.; (2000). Factors in Inhibition of DNA Repair and Cell Proliferation by Boronic Acid in Human Peripheral Blood Lymphocytes. Toxicon, 39(2), 155-159. https://doi.org/10.1016/S0041-0101(00)00072-6
Sawada, H., et al.; (2000). Factors in Inhibition of DNA Repair and Cell Proliferation by Boronic Acid in Human Peripheral Blood Lymphocytes. Toxicon, 39(2), 155-159. https://doi.org/10.1016/S0041-0101(00)00072-6
Shamsuddin, A.K., Khatoon, A., Hameed, B., & Amin, A. (2014). Optical density of the alkaline comet assay. Int. J. Pharm. Pharmaceut. Res., 6(1), 03-06. [Research gate]