

Food additives: Sodium benzoate, potassium sorbate, azorubine, and tartrazine modify the expression of NFκB, GADD45α, and MAPK8 genes

**B Raposa^{1,2}, R Pónusz³, G Gerencsér¹, F Budán¹, Z Gyöngyi¹, A Tibold¹,
D Hegyi¹, I Kiss¹, Á Koller⁴, T Varjas¹**

¹Institute of Public Health, Medical School, University of Pécs, Pécs, Hungary

²Institute of Human Nutrition and Dietetics, Faculty of Health Sciences, University of Pécs, Pécs, Hungary

³Institute of Health Insurance, Faculty of Health Sciences, University of Pécs, Pécs, Hungary

⁴Institute of Natural Sciences, University of Physical Education, Budapest, Hungary

Received: December 14, 2015

Accepted: July 5, 2016

It has been reported that some of the food additives may cause sensitization, inflammation of tissues, and potentially risk factors in the development of several chronic diseases. Thus, we hypothesized that expressions of common inflammatory molecules – known to be involved in the development of various inflammatory conditions and cancers – are affected by these food additives. We investigated the effects of commonly used food preservatives and artificial food colorants based on the expressions of NFκB, GADD45α, and MAPK8 (JNK1) from the tissues of liver. RNA was isolated based on Trizol protocol and the activation levels were compared between the treated and the control groups. Tartrazine alone could elicit effects on the expressions of NFκB ($p = 0.013$) and MAPK8 ($p = 0.022$). Azorubine also resulted in apoptosis according to MAPK8 expression ($p = 0.009$). Preservatives were anti-apoptotic in high dose. Sodium benzoate (from low to high doses) dose-dependently silenced MAPK8 expression ($p = 0.004$ to $p = 0.002$). Addition of the two preservatives together elicited significantly greater expression of MAPK8 at half-fold dose ($p = 0.002$) and at fivefold dose ($p = 0.008$). This study suggests that some of the food preservatives and colorants can contribute to the activation of inflammatory pathways.

Keywords: food additives, gene expression, inflammation, apoptosis, cancer

Introduction

Since the middle of the 20th century, the use of food additives became widespread and increasing in each food groups. Both producers and consumers have higher expectations for various food products, which also promote the use of food additives to improve their consistency, taste, appealing, color, and longer shelf life. It follows that most producers involved in food industry are using food additives to achieve the desired organoleptic characteristics of each product (16). Unfortunately, however these additives may have adverse health effects as recent studies demonstrated such side effects (13, 14).

For example, azorubine and tartrazine artificial colorants are still on the market, although there are reports showing that they may cause attention deficit and hyperactivity disorder in children and teenagers (3, 9). In addition, Amin et al. (1) detected altered kidney

Corresponding author: Bence Raposa

Institute of Human Nutrition and Dietetics, Faculty of Health Sciences, University of Pécs

Rét u. 4, Pécs H-7623, Hungary

Phone: +36 30 277 5693; Fax: +36 72 535 982; E-mail: bence.raposa@etk.pte.hu

and liver function and oxidative stress biomarkers after tartrazine and azorubine intake in male rats. According to their data, in the treated group alanine aminotransferase, aspartate aminotransferase, total protein, and albumin levels were significantly increased in comparison to the control group, especially at tenfold concentration exposure. Also, in liver homogenates, level of glutathione, *superoxide dismutase*, and catalase decreased, whereas malondialdehyde level increased at tartrazine and azorubine, thus they likely induce oxidative stress (1, 28).

Increased tissue concentrations of enzymes involved in the oxidative mechanisms indicate that these food colorants may intervene in the multi-step process of inflammation and carcinogenesis. Investigating DNA damage, Poul et al. (21) did not find significant increase in the number of micronuclei even at high dose of tartrazine in mice. Notwithstanding, tartrazine and azorubine increased the mRNA level of CYP1A1, which are involved in the metabolic activation of certain procarcinogenic substances in the liver of mice (21).

Preservatives protect food from spoiling and also increase shelf life. Among the preservatives, sodium benzoate and potassium sorbate are the most frequently used preservatives in foods. They can burden the liver, cause sensitization, and affect children's behavior (4). Clastogenic, mutagenic, and cytotoxic effects of the mentioned preservatives are proven *in vitro* on human lymphocytes and they may cause cancer (17, 25, 29).

Genes that are involved in carcinogenic processes, as early biomarkers of toxic and carcinogenic effect, would provide a better understanding of the underlying factors, regulatory pathways, and potential influencing effect on other factors (e.g., diseases, inflammations, etc.).

Nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) is a transcription factor, which can be activated by Toll-like receptors, Tumor Necrosis Factor alpha receptor, tyrosine kinase receptor, and adhesion receptors that mainly promotes cell survival and proliferation (5). It can be activated by many physical and biological interventions (e.g., irradiation, lymphotoxins, and free radicals, inter alia) and possess a wide range of receptor connections. NFκB stimulates the transcription of the anti-apoptotic processes that are necessary for the survival and proliferation of the cells (11). Growth arrest and DNA damage-inducible 45 alpha (GADD45α) is activated by oxidative stress and inhibition of NFκB stabilizes GADD45α mRNA (30). Further GADD45α mRNA accumulation is seen in response to genotoxic stress (20). Functionally, it stimulates DNA repair, apoptosis, and inhibits cells to enter S phase (24).

Another important molecule is mitogen-activated protein kinase 8 (MAPK8), which belongs to the family of c-Jun N-terminal kinases (JNKs). Formerly, its name was JNK1. This kinase is activated by different cell stimulations, and connects to immediate early gene expression in response to stimulus and environmental stress (2, 15, 19). We have selected MAPK8 activity because it is a specific and sensitive marker for the inflammatory pathways that we investigated (26). Resulting enhanced apoptosis, GADD45 genes has an activating effect on MAPK8 genes (27). Based on the above changes in the expressions of NFκB, GADD45α, and MAPK8, genes can be biomarkers of proliferative and/or anti-proliferative events.

Thus, in this study, we hypothesized that expressions of key genes in cell proliferation, apoptosis, and inflammation are affected by these food additives. In order to test this hypothesis, we examined the changes in the expression of NFκB, GADD45α, and MAPK8 genes on mRNA level in the liver of mice groups fed with tartrazine, azorubine,

sodium benzoate, and potassium sorbate to assess the proliferative and apoptotic impacts of these food additives.

Materials and Methods

Substance tested and their characteristics

Tartrazine (Sigma-Aldrich, Hungary) is a yellow color substance known as E102 or FD&C Yellow 5, which mostly consists of trisodium-1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-5-pyrazolone-3-carboxylate and a few by-colors (8). The calculated maximum daily exposure of tartrazine is 8.1 mg/kg bw/day for adults and 13.1 mg/kg bw/day for a typical 3-year-old child (9). The acceptable daily intake (ADI) is 7.5 mg/kg bw/day.

Azorubine (Medimpex, Hungary) is a red color substance known as Carmoisine, E122 or Food Red 3 Azorubin S, Brillant Carmoisine O, Acid Red 14, or C.I. 14720. Azorubine consists of essentially disodium-4-hydroxy-3-(4-sulfonato-1-naphthyl)-naphthalene-1-sulfonate. Usually, sodium salt of azorubine is used. Calcium and potassium salts of azorubine are also applied as a food coloring (7, 8).

The ADI is 4 mg/kg bw/day (8). Sodium benzoate (E211; Unik, Hungary) is the sodium salt of benzoic acid. The benzoic acid is an organic acid, which can be found in blueberries and other fruits; also in honey, yogurt, sour milk, and cheese (12). It is widely used as a preservative in the food, cosmetics, and pharmaceutical industries. Its ADI is 5 mg/kg bw/day (8).

The ADI of potassium sorbate (Sigma-Aldrich, Hungary) is 25 mg/kg bw/day (8, 12). The human equivalent doses were calculated for mice consuming 2 g of standard rodent feed daily and 12.3 was the conversion rate (22).

Animals used and treatments

The study was conducted on CD1 male and female mice ($n = 6$ /group; three females, three males) (6–8 weeks old, weighing 30–40 g). The animals were obtained from Experimental Department, where they were housed in standard polycarbonate cages (330 × 160 × 137 mm), bedded with shavings under laboratory conditions (20–22 °C, humidity 40–60%, 12-h light/dark photoperiod) and fed with standard rodent pellet (CRLT/n standard rodent pellet, Szindbád Kft., Gödöllő, Hungary) and water was provided *ad libitum*. The control group received regular rodent chow. For the treated groups, azorubine, tartrazine, sodium benzoate, and potassium sorbate were added to the regular chow. During the 42-day-long period, the animals were fed with different equivalent of human doses of these additives, administering alone or together to the chow (Table I). These represent a normal dose (onefold) and superdoses (fivefold and tenfold) of the additives. We relied on the manufacturer data regarding the composition of chow. The animals received similar amount of chow, which were consumed daily. The animal experiment was reviewed and approved by the local authorities (Committee on Research of the University of Pécs, permit number: BA02/2000-5/2015), and according to Hungarian animal protection laws in accordance with EU guidelines.

Sample collections and gene expression analysis

After the treatment period, samples were taken from the liver during autopsy after cervical dislocation. Based on Trizol protocol, we isolated RNA from the tissue (6). The gene expression (NF B, GADD45 α , and MAPK8) was determined with quantitative RT-PCR using SYBR Green protocol and ROSCHE Light Cycler 480 instrument. We designed the

Table I. Name and doses of colorants and preservatives

Colorants		Preservatives	
Material	Dose	Material	Dose
Tartrazine	92.5 mg/kg bw ^a	Sodium benzoate	30.8 mg/kg bw ^c
Tartrazine	925 mg/kg bw ^b	Sodium benzoate	308 mg/kg bw ^d
Azorubine	49.3 mg/kg bw ^a	Potassium sorbate	154 mg/kg bw ^c
Azorubine	493 mg/kg bw ^b	Potassium sorbate	1,540 mg/kg bw ^d
Tartrazine + azorubine	92.5 mg/kg bw + 49.3 mg/kg bw	Sodium benzoate + Potassium sorbate	30.8 mg/kg bw + 154 mg/kg bw
Tartrazine + azorubine	925 mg/kg bw + 493 mg/kg bw	Sodium benzoate + Potassium sorbate	308 mg/kg bw + 1540 mg/kg bw

^aOnefold human ADI equivalent dose in regular rodent feed; ^bTenfold human ADI equivalent dose in regular rodent feed; ^cHalf-fold human ADI equivalent dose in regular rodent feed; ^dFivefold human ADI equivalent dose in regular rodent feed

primers with Primer Express™ software (Applied Biosystems, Waltham, MA, USA). Forward and reverse primers were 5'AGTTGAGGGGACTTTCCAGGC3', 5'GCCTGG GAAAGTCCC CTCAACT3' (NF B); 5'GTGCTCAGCAAGGCTCGGA3', 5'GCTGCTCA ACGTAGACCCC3' (GADD45α); 5'GTTGCTCATAAACACAAACCTCC3', 5'GGGA TGGGT TATGTTTTCTAGAC3' (MAPK8); 5'AGGGCATATCCAACAACAACTT3', 5'GTTAAGCAGTAC AGCCCCAAA3' (hypoxanthine-guanine phosphoribosyltransferase 1, HPRT1). Then, we determined the relative gene expression with “comparative CT method” (ΔΔCt method, Applied Biosystems) compared to HPRT1.

Statistical analysis

Comparing the groups, we used ANOVA test followed by post-hoc analyzes. The level of statistical significance was determined at $p > 0.05$ with a 95% confidence interval. Statistical analyses were performed using SPSS 22 software package.

Results

Expression pattern of NFκB

In respect of the food dyes, NFκB showed significantly elevated expression compared to the control group at tenfold tartrazine ($p = 0.013$) and combined onefold dose of azorubine and tartrazine ($p = 0.014$). Data are shown in Fig. 1.

Sodium benzoate solely decreased NFκB expression at half-fold dose ($p = 0.041$). Sodium benzoate and potassium sorbate together rose NFκB at half-fold dose ($p = 0.006$) (Fig. 2).

Expression pattern of GADD45α

The artificial colorants investigated did not significantly modify the GADD45α expression (Fig. 3).

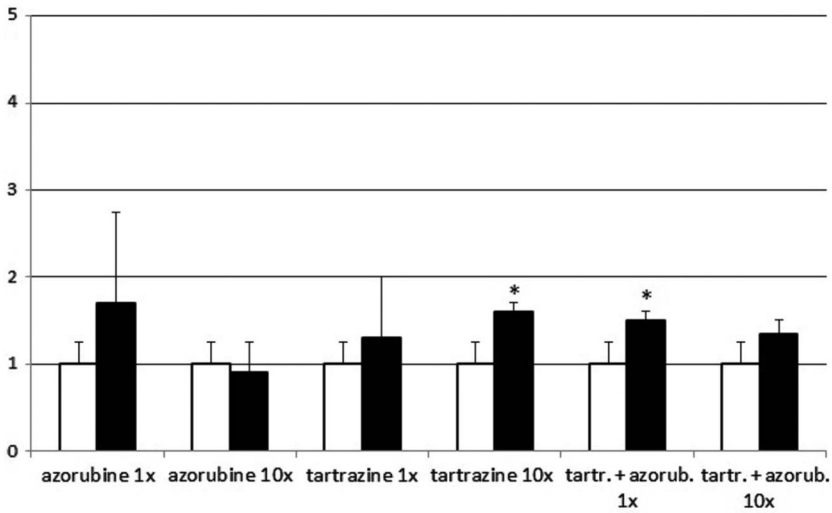


Fig. 1. Normalized expressions of NFκB (to HPRT1) in control animals (□) and after feeding the animals with food colorants (■). Data are mean ± SD ($n = 36$). Asterisks indicate the statistical compared to the control group ($p < 0.05$)

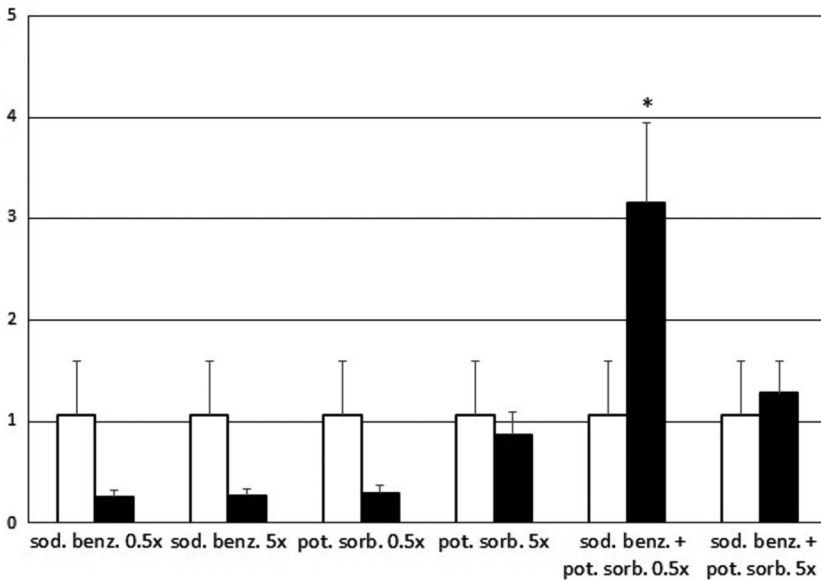


Fig. 2. Normalized expressions of NFκB (to HPRT1) in control animals (□) and after feeding the animals with preservatives (■). Data are mean ± SD ($n = 36$). Asterisk indicates the statistical significance compared to the control group ($p < 0.05$)

While sodium benzoate ($p = 0.034$) and potassium sorbate ($p = 0.013$) decreased GADD45α expression separately at half-fold dose; and at fivefold dose, potassium sorbate increased GADD45α expression ($p = 0.019$). Together, these substances raised GADD45α both at half-fold ($p = 0.034$) (Fig. 4).

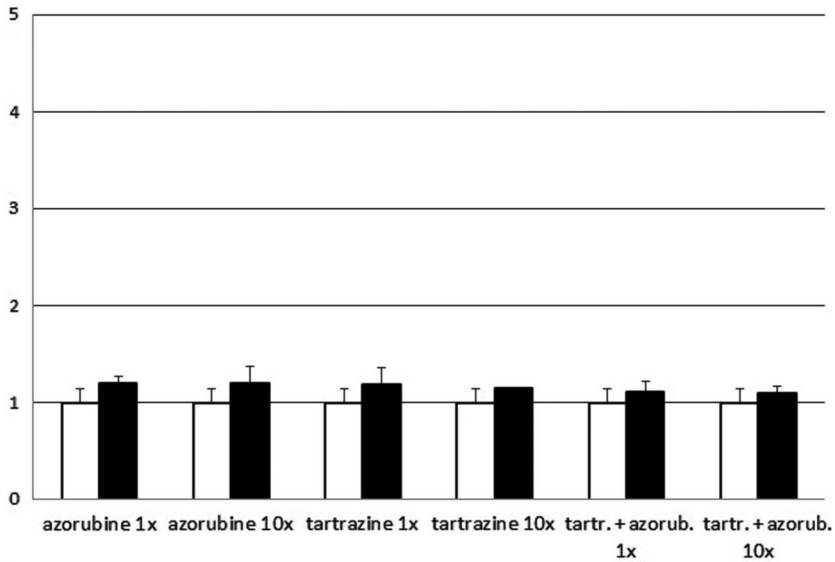


Fig. 3. Normalized expressions of GADD45 α (to HPRT1) in control animals (□) and after feeding the animals with food colorants (■). Data are mean \pm SD ($n = 36$)

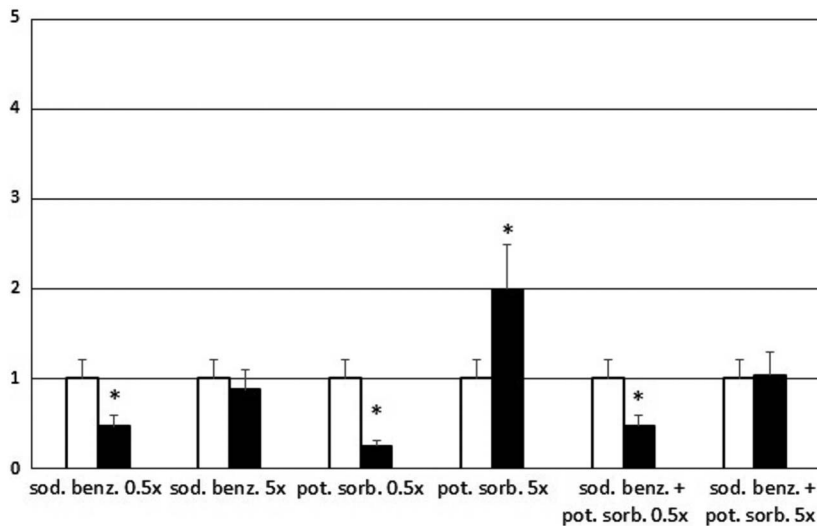


Fig. 4. Normalized expressions of GADD45 α (to HPRT1) in control animals (□) and after feeding the animals with preservatives (■). Data are mean \pm SD ($n = 36$). Asterisks indicate the statistical significance compared to the control group ($p < 0.05$)

Expression pattern of MAPK8

We found a significant rise of MAPK8 expression at onefold dose ($p = 0.009$) and at tenfold dose ($p = 0.028$) in azorubine-treated groups when compared to the control group. Tartrazine also increased the expression of MAPK8 at onefold dose ($p = 0.022$) (Fig. 5).

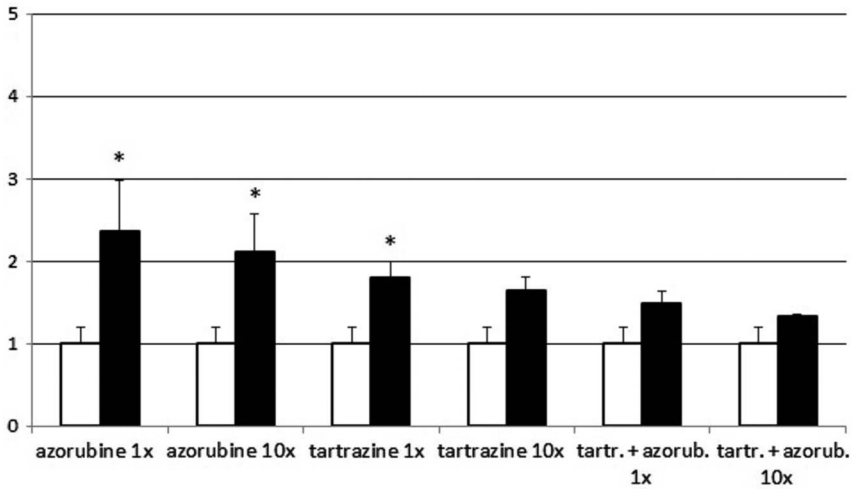


Fig. 5. Normalized expressions of MAPK8 (to HPRT1) in control animals (□) and after feeding the animals with food colorants (■). Data are mean \pm SD ($n = 36$). Asterisks indicate the statistical significance compared to the control group ($p < 0.05$)

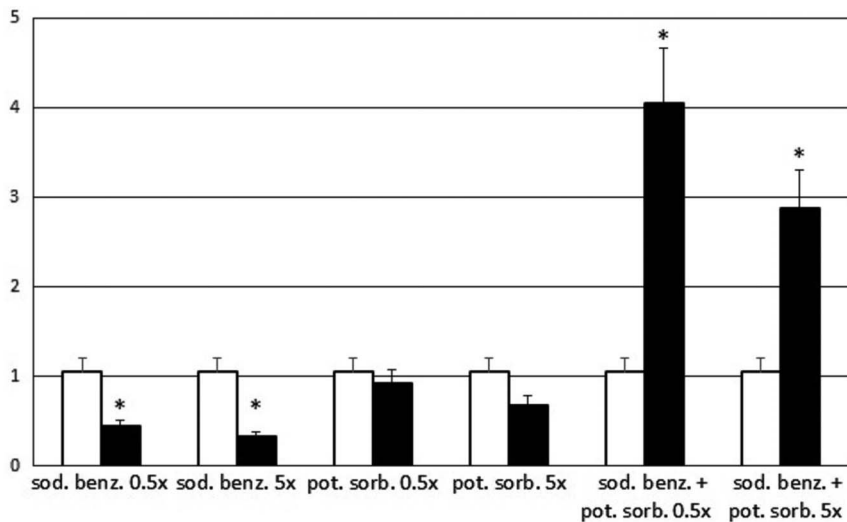


Fig. 6. Normalized expressions of MAPK8 (to HPRT1) in control animals (□) and after feeding the animals with preservatives (■). Data are mean \pm SD ($n = 36$). Asterisks indicate the statistical significance compared to the control group ($p < 0.05$)

Sodium benzoate dose-dependently silenced MAPK8 at half-dose ($p = 0.004$) and at fivefold dose ($p = 0.002$). Applying the two preservatives together at half-fold dose ($p = 0.002$) and at fivefold dose ($p = 0.008$), there was transcriptional activation of MAPK8 (Fig. 6).

Discussion

The salient findings of this study are: (1) the level of NF κ B and MAPK8 (compared with the control group) significantly increased in response to the higher doses of food additives, (2) the level of GADD45 α did not change in response to artificial food colorants, (3) the level of GADD45 α significantly increased in response to preservative doses, and (4) the increased sodium benzoate feeding dose-dependently silenced the expressions of MAPK8.

Apoptosis and food additives

In a previous study, Gao et al. (10) reported increased apoptotic characteristics of brain tissue of rats treated with 500 mg/kg/bw tartrazine. The findings of this study suggest a more complex phenomenon, namely – based on the NF κ B and MAPK8 expressions – tartrazine alone could contribute to apoptotic effects at low concentration and to anti-apoptotic effects at high concentration. In the present experiments, the doses were 41.2 and 412 mg/kg/bw translated to rat equivalent (10).

It follows that apoptotic feature of tartrazine is confirmed in liver at lower concentration in mice, but at high concentration we found rather an anti-apoptotic effect. Based on our results and responses of brain and liver to tartrazine (23), it would be important to conduct further experiments to assess whether tartrazine may contribute to apoptotic or anti-apoptotic effects in human organs and to map the dose-efficacy pattern as well (e.g., using *in vitro* cell cultures, etc.).

Although azorubine, utilized alone, increased MAPK8 expression at remarkably low and high concentrations, the expression of the other two genes did not change. Thus, azorubine elicited a weaker apoptotic stimulus than tartrazine. Addition of a mixture of dyes showed anti-apoptotic effect at low concentration based on NF κ B expression.

Intriguingly, dyes investigated did not significantly modify the GADD45 α expression. Sodium benzoate reduced the expression of all the examined genes at low concentration, but at high concentration anti-apoptotic feature was present based on MAPK8 expression.

Also, it has been observed that sodium benzoate has beneficial effects in schizophrenia, which could be due to MAPK8 activation leading to the down regulation of apoptotic activity (13). Potassium sorbate was anti-apoptotic at low concentration, but it seemed to activate apoptotic or cell cycle arrest processes at high concentration based on GADD45 α expression (24). Mamur et al. (17) also described that potassium sorbate may cause cell cycle arrest. It is of note that combined administration did not yield conclusive findings. However, our results confirm that colorants and preservatives increase the mitotic index and their application can be cytotoxic and mutagenic (21, 29). The elevated expression of MAPK8 also indicates that the tested substances can have a significant impact on tumor proliferation and some transcription factors (18).

Conclusion

On the basis of our findings, we suggest that some of the food additives and colorants can contribute – in a dose-dependent manner – to the activation of inflammatory pathways favoring the development of cancers, further emphasizing that it would be preferable to reduce the human intake of preservatives and artificial dyes.

REFERENCES

1. Amin KA, Abdel Hameid IH, Abd Elsttar AH: Effect of food azo dyes tartrazine and carmoisine on biochemical parameters related to renal, hepatic function and oxidative stress biomarkers in young male rats. *Food Chem. Toxicol.* 48, 2994–2999 (2010)
2. Banning A, Regenbrecht CRA, Tikkanen R: Increased activity of mitogen activated protein kinase pathway in flotillin-2 knockout mouse model. *Cell Signal.* 26, 198–207 (2014)
3. Bateman B, Warner JO, Hutchinson E, Dean T, Rowlandson P, Gant C, Grundy J, Fitzgerald C, Stevenson J: The effects of a double blind, placebo-controlled, artificial food colourings and benzoate preservative challenge on hyperactivity in a general population sample of preschool children. *Arch. Dis. Child.* 89, 506–551 (2004)
4. Brahmachari S, Pahan K: Sodium benzoate, a food additive and a metabolite of cinnamon, modifies T-cells at multiple steps and inhibits adoptive transfer of experimental allergic encephalomyelitis. *J. Immunol.* 179, 275–283 (2007)
5. Brasier AR: The NF- κ B regulatory network. *Cardiovasc. Toxicol.* 6, 111–130 (2006)
6. Chequer FM, Venâncio VP, Bianchi ML, Antunes LM: Genotoxic and mutagenic effects of erythrosine B, a xanthene food dye, on HepG2 cells. *Food Chem. Toxicol.* 50, 3447–3451 (2012)
7. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159 (1987)
8. European Parliament and of the Council [EC]: Regulation No. 1333/2008: establishing a common authorisation procedure for food additives, food enzymes and food flavourings. *OJ. L.* 354, 1–33 (2008)
9. European Food Safety Agency [EFSA]: Assessment of the results of the study by McCann et al. (2007) on the effects of some colours and sodium benzoate on children's behaviour. *EFSA J.* 660, 1–53 (2008)
10. Gao Y, Li C, Yin H, An X, Jin H: Effect of food azo dye tartrazine on learning and memory functions in mice and rats, and the possible mechanisms involved. *J. Food Sci.* 76, 125–129 (2011)
11. Gilmore TD: Introduction to NF- κ B: players, pathways, perspectives. *Oncogene* 25, 6680–6684 (2006)
12. Görena AC, Bilsela G, Şimşek A, Bilsela M, Akçadağ F, Topala K, Ozgen H: HPLC and LC-MS/MS methods for determination of sodium benzoate and potassium sorbate in food and beverages: performances of local accredited laboratories via proficiency tests in Turkey. *Food Chem.* 175, 273–279 (2015)
13. Jarskog LF: Apoptosis in schizophrenia: pathophysiologic and therapeutic considerations. *Curr. Opin. Psychiatry* 19, 307–312 (2006)
14. Kitano K, Fukukawa T, Ohtsuji Y, Masuda T, Yamaguchi H: Mutagenicity and DNA-damaging activity caused by decomposed products of potassium sorbate reacting with ascorbic acid in the presence of Fe salt. *Food Chem. Toxicol.* 40, 1589–1594 (2002)
15. Klock A, Herrmann BG: Cloning and expression of the mouse dual-specificity mitogen-activated protein (MAP) kinase phosphatase Mkp3 during mouse embryogenesis. *Mech. Develop.* 116, 243–247 (2002)
16. Leathwood PD, Richardson DP, Sträter P, Todd PM, van Trijp HC: Consumer understanding of nutrition and health claims: sources of evidence. *Br. J. Nutr.* 98, 474–484 (2007)
17. Mamur S, Yüzbaşıoğlu D, Ünal F, Yılmaz S: Does potassium sorbate induce genotoxic or mutagenic effects in lymphocytes? *Toxicol. In Vitro* 24, 790–794 (2010)
18. Mielke K, Brecht S, Dorst A, Herdegen T: Activity and expression of JNK1, p38 and ERK kinases, c-Jun N-terminal phosphorylation, and c-jun promoter binding in the adult rat brain following kainate-induced seizures. *Neuroscience* 91, 471–483 (1999)
19. Miotto B, Struhl K: JNK1 phosphorylation of Cdt1 inhibits recruitment of HBO1 histone acetylase and blocks replication licensing in response to stress. *Mol. Cell* 7, 62–71 (2011)
20. Peretz G, Bakhrat A, Abdu U: Expression of the *Drosophila melanogaster* GADD45 homolog (CG11086) affects egg asymmetric development that is mediated by the c-Jun N-terminal kinase pathway. *Genetics* 177, 1691–1702 (2007)
21. Poul M, Jarry G, Elhkim MO, Poul JM: Lack of genotoxic effect of food dyes amaranth, sunset yellow and tartrazine and their metabolites in the gut micronucleus assay in mice. *Food Chem. Toxicol.* 47, 443–448 (2009)
22. Reagan-Shaw S, Nihal M, Ahmad N: Dose translation from animal to human studies revisited. *FASEB J.* 22, 659–661 (2007)
23. Sasaki YF, Kawaguchi S, Kamaya A, Ohshita M, Kabasawa K, Iwama K, Taniguchi K, Tsuda S: The comet assay with 8 mouse organs results with 39 currently used food additives. *Mutat. Res.* 519, 103–119 (2002)
24. Sheikh MS, Hollander MC, Fornace AJ: Role of Gadd45 in apoptosis. *Biochem. Pharmacol.* 59, 43–45 (2000)

25. Soares BM, Araújo TM, Ramos JA, Pinto LC, Khayat BM, De Oliveira Bahia M, Montenegro RC, Burbano RM, Khayat AS: Effects on DNA repair in human lymphocytes exposed to the food dye tartrazine yellow. *Anticancer Res.* 35, 1465–1474 (2015)
26. Solinas G, Vilcu C, Neels JG, Bandyopadhyay GK, Luo JL, Naugler W, Grivennikov S, Wynshaw-Boris A, Scadeng M, Olefsky JM, Karin M: JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. *Cell Metab.* 6, 386–397 (2007)
27. Takekawa M, Saito H: A family of stress-inducible GADD45-like proteins mediate activation of the stress-responsive MTK1/MEKK4 MAPKKK. *Cell* 95, 521–530 (1998)
28. Tsuda S, Murakami M, Matsusaka N, Kano K, Taniguchi K, Sasaki YF: DNA damage induced by red food dyes orally administered to pregnant and male mice. *Toxicol. Sci.* 61, 92–99 (2001)
29. Türkoğlu S: Genotoxicity of five food preservatives tested on root tips of *Allium cepa* L. *Mutat. Res.* 626, 4–14 (2007)
30. Zheng X, Zhang Y, Chen Y, Castranova V, Shi X, Chen F: Inhibition of NF- κ B stabilizes gadd45 α mRNA. *Biochem. Biophys. Res. Commun.* 329, 95–99 (2005)