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In Vivo Comet Assay of Food Additives' Combinations and their Effects on Biochemical Parameters in Albino Rats

Salah Abo Raya [1], Ahmed M. Aboul-Enein [2], Mohamed M.A. El-Nikeety [1], Rasha S. Mohamed [3], Waleed M. A. Abdelwahid [4,*]

[1] Food Science Department, Faculty of Agriculture, Cairo University, Giza, Egypt; aborayasalah1947@yahoo.com (S.A.R.); mnoketis50@yahoo.com (M.M.A.E-N.);
[2] Biochemistry Department, Faculty of Agriculture, Cairo University Giza, Egypt; aboul.enein1@gmail.com (A.M.A-E.);
[3] Nutrition and Food Sciences Department, National Research Centre, Dokki, Giza, Egypt; smarasha2005@yahoo.com (R.S.M.);
[4] National Food Safety Authority, Cairo, Egypt; wsa_mostafa@hotmail.com (W.M.A.A.);
* Correspondence: wsa_mostafa@hotmail.com;

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Abstract: Although the safety of food additives had been assessed individually, these permitted additives may be unsafe if used together; this study was piloted to assess the safety of various food additive mixtures. Fifty male Albino rats - Wistar strain (4 weeks old) were distributed into 10 groups, the first group orally administered distilled water, the other nine groups orally administered different mixtures of food additives at NOAEL dosage for each food additive for 30 days. Haemoglobin, malondialdehyde, kidney functions, activities of AST, ALT, and ALP. Levels of bilirubin, total protein, and albumin were also determined. Assessment of the genotoxic effect using *in vivo* alkaline comet assay was performed in the brain, liver, and kidney tissues. The results indicated significant Hb concentration reduction was recorded by all studied food additives' combination compared to the control group. With the number of additives increases the Hb, total serum protein and albumin contents were significantly (p <0.05) decreased; in contrast, there was an increase in MDA, urea, creatinine, liver function enzyme activity, and bilirubin levels. Also, the examined food additives' combinations exhibited genotoxic activities with different degrees compared to control rats in the brain, kidney, and liver, with the number of additives increases the genotoxic effect increased.

Keywords: Comet assay; food additives' combinations; biochemical parameters; genotoxicity; ADI; NOAEL; food safety; haemoglobin (Hb).

1. Introduction

The risks associated with food, mainly, affect the consumer`s rights of health and safety. Food additives such as colorants, sweeteners, and preservatives are important for food processing and preservation [1]. Some food additives exhibited complete safety and absence of bone marrow genotoxicity and comet assay of liver and stomach [2] and [3,4]. On the other side, some food additives exhibited neurotoxic [5], immunotoxic [6], and teratogenic [7] effects. Some food additives can lead up to some health problems and can cause various allergies and conditions, such as attention deficit disorder and hyperactivity, in some individuals who are susceptible to specific chemicals. Also, hay fever, asthma, vomiting,

rashes, headaches, tight chest, hives, and aggravating of eczema are among conditions caused by some food additives [8]. Acesulfame K (E 950) is an artificial sweetener with an acceptable daily intake (ADI) of 0-15 mg/kg bw/day [9]**.** Allura Red AC (E129) is a food colorant with ADI of 0-7 mg/kg bw/day [10]. Ammonia caramel (E 150c) is a food colourant with ADI of 0-200 mg/kg bw/day [11,12]. Brown HT / Chocolate brown HT (E155) is a food colorant. It was previously evaluated by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1977 and the EU Scientific Committee for Food (SCF) in 1984. JECFA established an ADI of 0-1.5 mg/kg bw/day, while the SCF recognized an ADI of 0-3 mg/kg bw/day [13]. Azorubine/Carmoisine (E 122) is a permitted azo dye as a food additive and has previously been assessed by JECFA and SCF, the two committees established an ADI of 0-4 mg/kg bw/day [14,15]. Fast Green FCF (E143) is a food colorant with ADI of 0–25 mg/kg bw/day [16]. Glutamic acid and glutamates (E 620–625) are flavor enhancers, JECFA established a group of ADI 'not specified' for glutamic acid and its salts, but European Food Safety Authority`s (EFSA) Panel on Food Additives and Nutrient Sources added to food recognized a group ADI of 30 mg/kg bw/day, expressed as glutamic acid, for glutamic acid and glutamates (E 620–625) [17]. Quinoline Yellow (E104) is a food colorant with ADI of 0–3 mg/kg bw/day [18]. Sodium benzoate (E211) is a preservative with ADI of 0-5 mg/kg bw/day [19]. Sodium nitrite (E250) is used as a preservative and color retention agent with ADI of 0-0.07 mg/kg bw/day [20]. Sucralose is an artificial sweetener with ADI of 0-15 mg/kg bw/day [21]. Sunset Yellow FCF (E110) is a food colorant with ADI of 0–4 mg/kg bw/day [22]. Tartrazine (E102) is a food colorant with ADI of 0–10 mg/kg bw/day [23]. The acceptable daily intake (ADI) for any food additive is determined by dividing it's No-Observed Adverse Effect Level (NOAEL) by appropriate safety or uncertainty factor; the default safety (uncertainty) factor is 100 [24,25,26].

The *in vivo* alkaline comet assay, a rapid and sensitive assay which reveals DNA damage as strand breaks, is especially pertinent to an assessment of the genotoxic hazards of xenobiotics, as its responses mirror the *in vivo* absorption, tissue distribution, metabolism, and secretion of chemicals in addition to DNA repair process [27]. The regulatory guidelines of the International Conference on Harmonisation [28] and EFSA [29] recommended the *in vivo* comet assay for evaluating product safety.

Most studies on the consequences of food additives have focalized on studying them individually, whereas in fact, these additives are used in a combined form, especially that each food category may contain preservatives along with coloring agents, artificial sweeteners, emulsifiers, anti-caking, acidulants, etc. So, due to consuming these food additives together, these permitted food additives may be unsafe or due to the presence of additive-additive interaction; it's necessary to appraise the combination effect of various food additive mixtures on food safety. The current study was conducted to estimate the genotoxicity of food additives' combinations using *in vivo* comet assay and to evaluate their effects on biochemical parameters.

2. Materials and Methods

2.1. Chemicals.

Allura Red AC (E129), Carmoisine (E122), Quinoline Yellow (E104), Sunset Yellow FCF (E110), and Tartrazine (E102) were purchased from ROHA Dyechem Pvt. Ltd., India. Ammonia caramel (E150c) and Brown HT (E155) were purchased from Raj Bakers Field for

Food Industry, India. Sucralose (E955) was purchased from Anhui Jinhe Industrial Co., Ltd., China. Monosodium L-glutamate (E621) (MSG) was purchased from Loba Chemie Pvt. Ltd., India. Fast Green FCF (E143) was purchased from Oxford Lab Chem. Co., India. Acesulfame K (E 950) was purchased from Vitasweet Co., Ltd., China. Sodium benzoate (E211) was purchased from S.D. Fine-Chem. Ltd., India, and Sodium nitrite (E250) was purchased from Bio Basic Canada Inc., Canada.

Low-melting-point agarose, normal-melting-point agarose, and ethidium bromide were purchased from Fermentas (Glen Burnie, MD, USA). All other chemicals and reagents were of analytical grade and obtained from standard commercial suppliers.

2.2. Experimental animals.

Fifty male Albino Wistar rats of 4 weeks old and weight 65.15±6.8 g (as mean±SEM) were obtained from the animal house of National Research Centre, Giza, Egypt. Rats were initially fed a standard balanced diet and maintained individually in stainless steel cages under controlled conditions (22 ± 2 °C, 55 ± 10 % relative humidity and 12-hourly cycling of light and dark) for 5 days to be accommodated with laboratory conditions before being treated. Water and food were given *ad-libtium*.

2.3. Selection of food additives.

On May 31, 2015, the Egyptian Minister of Health issued Decree 204/2015 regarding permitted food additives [30], it is highly compatible with Regulation (EC) No 1333/2008 of the European Parliament and of the Council on food additives, revision 2018 [31], and with the codex standard 192/1995 general standard for food additives, revision 2019 [32]. The Decree divides food into many categories, and it lists all usage concentrations of permitted food additives in each food category. Food categories that children favorite to consume were selected, in each selected food category, food additives with more safety debate were selected. The safety of selected mixtures of food additives was estimated. In the worst conditions, people may consume the selected thirteen food additives on the same day; so, the safety of the thirteen food additives, together, were estimated. Food categories and selected food additives are shown in Table 1.

2.4. Experimental design.

The experiment was conducted as recommended by Organization for Economic Cooperation and Development (OECD) Guidelines [33]; Rats were randomly assigned to 10 groups, 5 rats for each group; the control group was orally administrated with 1 ml/rat/day of distilled water (force feed tube) without additives. Other groups (G1: G9) were orally administrated the tested food additive solutions, as shown in Table 1, groups were orally administrated with 1ml of solutions /rat/ day. The doses for the groups from G1 to G8 were NOAEL of food additives [ADI for each food additive multiplied by the default uncertainty factor (100)] as mg/kg bw/day. Meanwhile, doses for G9 didn`t exceed 1000 mg/kg bw/day, although NOAEL for some food additives is more than 1000 mg/kg bw/day; because it was so difficult to dissolve all these quantities of food additives together.

Animals were orally administrated with the solution by stomach tube daily for 30 days. Rats were fed on balanced diet (10% protein, 10% corn oil, 3.5% salt mixture, 1% vitamin mixture, 5% cellulose, 10% sucrose and 60.5% corn starch) which formulated in accordance

with AIN-93 [34]. During the experiment, body weight and food intake were recorded weekly. After 30 days (end of the study), total food intake, body weight gain, and feed efficiency ratio (Bodyweight gain/total food intake) were calculated.

Table 1. Selected food categories, combinations of food additives, and doses.

Food category	combination of food additives	ADI (mg/kg bw/day)	Groups of rats	Dose (mg/kg bw/day) (ADI ×100)
Non-carbonated water-based flavored drinks (Red color)	Sodium benzoate	5	G1	500
	Carmoisine	4		400
	Sucralose	15		1500
Non-carbonated water-based flavored drinks (Green color)	Sodium benzoate	5	G2	500
	Fast Green FCF	25		2500
	Sucralose	15		1500
Non-carbonated water-based flavored drinks (Yellow color)	Sodium benzoate	5	G3	500
	Tartrazine	10		1000
	Sucralose	15		1500
Confectionery and chewing gum	Sodium benzoate	5	G4	500
	Fast Green FCF	25		2500
	Allura Red AC	70		700
	Sunset yellow FCF	4		400
	Acesulfame K	15		1500
Heat-treated processed comminuted (minced) meat, poultry, and game products include luncheon meat	Monosodium L-glutamate	30	G5	3000
	Sodium nitrite	0.07		7
Pre-cooked pasta and noodles and like products	Monosodium L-glutamate	30	G6	3000
	Sodium benzoate	5		500
	Sunset Yellow FCF	4		400
Snacks - potato, cereal, flour or starch-based (from roots and tubers, pulses and legumes)	Sucralose	15	G7	1500
	Sodium benzoate	5		500
	Ammonia caramel	200		20000
Edible ices, including sherbet and sorbet	Fast Green FCF	25	G8	2500
	Quinoline Yellow	3		300
	Carmoisine	4		400
	Brown HT	1.5		300
	Sucralose	15		1500
The worst condition (consuming the thirteen food additive on the same day)	Acesulfame K	15	G9	1000*
	Allura Red AC	7		700
	Ammonia caramel	200		1000 *
	Brown HT	3		300
	Carmoisine	4		400
	Fast Green FCF	25		1000*
	Monosodium L-glutamate	30		1000*
	Quinoline Yellow	3		300
	Sodium benzoate	5		500
	Sodium nitrite	0.07		7
	Sucralose	15		1000*
	Sunset Yellow FCF	4		400
	Tartrazine	10		1000

G1: G8 refers to groups of rats that were orally administrated mixtures of food additives, which are permitted in the corresponding food categories in the table, while G9 refers to the group of rats which orally administrated a mixture of the selected thirteen food additives together as the worst condition. *Doses were 1000 mg/kg bw/day only, although these food additives` ADIs are more than 100 mg/kg bw/day; because it was so difficult to dissolve all these quantities of food additives together.

2.5. Analysis of blood and tissues.

Blood samples were collected from the retro-bulbar plexus of the median canthus of the eyes of the rats using sterile microhematocrit capillary tubes from all rats after an overnight fast. A portion of the whole blood was analyzed for hemoglobin (Hb) concentration, according to [35]. The remaining blood was centrifuged, and the serum was analyzed for levels of malondialdehyde (MDA) to determine lipid peroxidation according to [36], creatinine, and urea depending on [37,38] in succession as indicators of kidney functions. The activities of aspartate transaminase (AST) and alanine transaminase (ALT) were determined according to [39]. The activity of alkaline phosphatase (ALP) was determined according to [40]. The levels of bilirubin, total protein, and albumin were determined according to [41,42,43] in succession. After blood sampling, rats were dissected, and the brain, liver, and kidney were immediately separated from each rat and weighed, and the relative organ's weight to body weight was calculated and then subjected to the *in vivo* alkaline comet assay. All animal procedures have been carried out according to the Medical Research Ethics Committee, National Research Centre, Cairo, Egypt, and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals [44].

2.6. In vivo comet assay.

In vivo Comet assay was performed referring to the protocol developed by [45], with minor modifications. The liver, brain, and kidney cells of each rats' group were mixed with low-melting-point agarose (ratio of 1:10v/v), then pipetted to precoated slides with normal-melting-point agarose. The slides were kept flat at 4°C for 30 min in a dark environment. The third layer of low melting point agarose was then pipetted on slides, left to solidify at for 30 min 4°C. The slides were transferred to a pre-chilled lysis solution, kept for 60min at 4°C. After that, slides were immersed in freshly prepared alkaline unwinding solution at room temperature in the dark for 60 min. Slides were subjected to electrophoresis run at 0.8 V/cm, 300mAmps at 4°C for 30 min. The slides were rinsed in a neutralizing solution followed by immersion in 70% ethanol and then air-dried. Ethidium bromide was used for slides stain then and visualized by using a Zeiss epifluorescence microscope (510–560 nm, barrier filter 590 nm) with a magnification of ×400. 100 cells per animal, 3 replicates, were scored then analyzed with DNA damage analysis software (Comet Score, TriTek corp., Sumerduck, VA22742).

2.7. Statistical analysis.

Data on body weight gain, relative organ weight, and blood analyses were statistically analyzed using the one-way analysis of variance ANOVA followed by Duncan's test. Data of comet assay were analyzed using the General Linear Models (GLM) procedure of the Statistical Analysis System [46], followed by Scheffé-test to assess significant differences between groups. All results are expressed as mean±SEM. All statements of significance were based on the probability of $P < 0.05$.

3. Results and Discussion

Although the individual food additive utilized at its NOAEL is expected to be relatively safe for Albino Wistar rats, but the effects of food additives' combinations at these levels are not clear.

3.1. Effect of food additives' combinations on rats' growth performance parameters.

The calculated total food intake for all treated rats was significantly less than that of control rats (Table 2), and thus the body weight gain for most treated rats was significantly less than that of control rats, but rats of groups G8 and G9 recorded the lowest total food intake, respectively, and the lowest body weight gain. The feed efficiency ratio (Table 2) for all treated rats was significantly less than that of control rats, but rats of groups G8 and G9 recorded the lowest feed efficiency ratio, respectively.

Table 2. Growth performance parameters of rats treated with different combinations of food additives.

Groups of rats (n=5)	Initial B.W. (g)	Final B.W. (g)	B.W. gain (g)	Total feed intake (g)	Feed Efficiency Ratio
Control	$65.40^{a}\pm3.12$	$119.60^{e}\pm8.31$	$54.20^{f}\pm7.79$	$425.00^{f}\pm8.93$	$0.13^{e}\pm0.02$
G1	$65.00^{a}\pm3.40$	$109.80^{de}\pm5.85$	$44.80^{ef}\pm3.82$	$386.00^{e}\pm10.57$	$0.12^{de}\pm0.01$
G2	$65.40^{a}\pm3.74$	$95.00^{abcd}\pm4.73$	$29.60^{abcde}\pm4.80$	$378.80^{de}\pm10.47$	$0.08^{abcde}\pm0.01$
G3	$65.20^{a}\pm3.30$	$109.60^{de}\pm6.18$	$44.40^{def}\pm7.05$	$384.20^{e}\pm12.56$	$0.12^{de}\pm0.02$
G4	$64.80^{a}\pm3.33$	$91.20^{abc}\pm3.75$	$26.40^{abc}\pm3.02$	$373.60^{de}\pm3.38$	$0.07^{abcd}\pm0.01$
G5	$65.40^{a}\pm3.33$	$89.60^{abc}\pm4.43$	$24.20^{ab}\pm6.98$	$368.00^{de}\pm10.77$	$0.06^{abc}\pm0.02$
G6	$65.00^{a}\pm5.06$	$92.00^{abc}\pm3.63$	$27.00^{abcd}\pm3.08$	$310.80^{ab}\pm11.72$	$0.09^{abcde}\pm0.01$
G7	$65.20^{a}\pm2.13$	$89.80^{abc}\pm3.26$	$24.60^{ab}\pm1.43$	$350.00^{cd}\pm7.80$	$0.07^{abcd}\pm0.01$
G8	$65.00^{a}\pm2.98$	$83.40^{ab}\pm4.18$	$18.40^{a}\pm4.96$	$303.00^{ab}\pm7.03$	$0.06^{ab}\pm0.02$
G9	$65.40^{a}\pm4.16$	$80.20^{a}\pm3.56$	$14.80^{a}\pm4.23$	$295.80^{a}\pm7.58$	$0.05^{a}\pm0.02$

G1: G9 refers to groups of rats that were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM

It was obvious (Table 3) that food additives' combinations didn't lead to significant ($p > 0.05$) changes in the brain weight in comparison with control rats, whereas significant ($p < 0.05$) elevations in kidney and liver weight of rats treated with different food additives' combinations, more extremely rats of G8 and G9 groups.

Although the artificial sweeteners such as sucralose and acesulfame k make humans and laboratory animals tend to overeat products containing these sweeteners [47] but the combined food additives in the current study led to a decline in the total food intake, which resulted in a reduction in both body weight gain and food efficiency ratio. This decline in the total food intake may indicate the association between combined food additives consumption and loss of appetite. Also, the reduction of body weight may be attributed to the hyperactivity associated with food additives intake, which confirmed by [48], who emphasized that food colors induce hyperactivity in children.

Table 3. Relative organs' weights of rats were treated with different combinations of food additives.

Groups of rats (n=5)	Relative brain's weight	Relative kidney's weight	Relative liver's weight
Control	$1.35^{a}\pm0.03$	$0.88^{a}\pm0.06$	$3.62^{a}\pm0.08$
G1	$1.34^{a}\pm0.05$	$0.92^{ab}\pm0.08$	$3.69^{a}\pm0.39$
G2	$1.26^{a}\pm0.09$	$0.95^{ab}\pm0.05$	$3.76^{ab}\pm0.41$
G3	$1.29^{a}\pm0.08$	$0.95^{ab}\pm0.04$	$3.76^{ab}\pm0.40$
G4	$1.27^{a}\pm0.08$	$1.01^{ab}\pm0.02$	$4.22^{abcd}\pm0.17$
G5	$1.14^{a}\pm0.05$	$1.11^{bc}\pm0.07$	$4.95^{cde}\pm0.13$

Groups of rats (n=5)	Relative brain's weight	Relative kidney's weight	Relative liver's weight
G6	$1.24^{a}\pm0.08$	$1.11^{bc}\pm0.03$	$4.29^{abcd}\pm0.31$
G7	$1.22^{a}\pm0.08$	$1.06^{abc}\pm0.08$	$4.22^{abcd}\pm0.35$
G8	$1.20^{a}\pm0.11$	$1.24^{c}\pm0.09$	$5.57^{e}\pm0.17$
G9	$1.19^{a}\pm0.05$	$1.44^{d}\pm0.02$	$5.04^{de}\pm0.38$

G1: G9 refers to groups of rats that were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM.

3.2. Effect of food additives' combinations on Hb, MDA, and kidney functions.

Table 4 portrays the effect of food additives' combinations on Hb, MDA, as well as kidney functions (urea and creatinine). Regarding Hb, significant reductions were recorded by all studied food additives' combinations in comparison to the control group; rats in groups G4, G5, G8 and G9, respectively, recorded the lowest Hb levels. Rats treated with all studied food additives' combinations showed elevations in MDA when compared to the control group; rats' groups G5, G8, and G9 were the highest levels of MDA. As for kidney functions, orally administration of food additives' combinations, more extremely G5, G8, and G9, respectively, produced significant elevations in either urea or creatinine compared to the control group.

Table 4. Hb, MDI, and kidney function of rats were treated with different combinations of food additives.

Groups of rats (n=5)	Hb (g/dl)	MDA (nmol/ml)	Urea (mg/dl)	Creatinine (mg/dl)
Control	$13.41^{h}\pm0.53$	$9.22^{a}\pm0.70$	$27.52^{a}\pm0.60$	$0.41^{a}\pm0.03$
G1	$11.26^{fg}\pm0.36$	$9.89^{ab}\pm0.58$	$28.20^{a}\pm0.87$	$0.73^{b}\pm0.06$
G2	$9.86^{bcd}\pm0.21$	$10.96^{abc}\pm0.59$	$30.54^{abcde}\pm1.48$	$0.99^{bc}\pm0.13$
G3	$11.03^{efg}\pm0.33$	$12.00^{cd}\pm0.74$	$30.24^{abcd}\pm0.97$	$0.82^{b}\pm0.06$
G4	$9.41^{b}\pm0.38$	$13.08^{def}\pm0.56$	$31.76^{bcdef}\pm1.05$	$1.10^{cd}\pm0.14$
G5	$9.22^{ab}\pm0.30$	$16.60^{hij}\pm0.62$	$34.47^{fg}\pm0.79$	$1.31^{def}\pm0.08$
G6	$9.62^{bc}\pm0.27$	$14.49^{efg}\pm1.25$	$33.47^{efg}\pm1.06$	$1.18^{cde}\pm0.12$
G7	$9.74^{bcd}\pm0.29$	$15.02^{fgh}\pm0.78$	$33.26^{defg}\pm1.12$	$1.21^{cde}\pm0.11$
G8	$9.24^{ab}\pm0.25$	$17.11^{ij}\pm0.36$	$35.63^{g}\pm0.72$	$1.42^{ef}\pm0.09$
G9	$8.25^{a}\pm0.23$	$18.22^{j}\pm0.41$	$38.79^{h}\pm0.89$	$1.57^{f}\pm0.13$

G1: G9 refers to groups of rats that were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM

Food additives can produce reactive oxygen species (ROS) in various organs, and induced oxidative stress turned out through elevation of lipid peroxidation and reduction of catalase, superoxide dismutase, and glutathione. This was confirmed by several studies conducted on different food additives, for example, sodium benzoate [49], monosodium glutamate [50], sodium nitrite [51], tartrazine, and carmoisine [52]. The reduction of Hb and disturbance of kidney and liver functions occurred upon administration of combined food additives in the present study can be attributed to the oxidative stress and elevation of lipid peroxidation as mentioned in the previous study [53] since oxidative stress and elevated lipid peroxidation are associated with hemolysis of red blood cells also the oxidative alteration of lipids and proteins can disrupt membrane integrity allowing the loss of enzyme molecules from the epithelial cell lining. All combinations of studied food additive reduced Hb level under 12 g/dl, induced hemoglobinemia [54], the combination of all selected food additives in G9 made the worst impact. Non-nutritive sweeteners altered appetite, gut hormonal secretion,

adipogenesis, glucose absorption, kidney function, in vitro insulin secretion, and microbiome dysbiosis of gut bacteria, and associated metabolic increased body mass index, increased risk of obesity, and abnormal liver function tests [55]. Azo dyes (as tartrazine, sunset yellow, carmoisine, allura red) at ADI levels adversely affect and alter biochemical markers of brain tissue and cause oxidative damage [56].

3.3. Effect of food additives' combinations on liver functions.

Table 5. The liver function of rats was treated with different combinations of food additives.

Groups of rats (n=5)	AST (U/l)	ALT (U/l)	ALP (U/l)	γ-GT (U/l)
Control	$31.56^{a}\pm1.00$	$19.08^{a}\pm1.22$	$190.10^{a}\pm2.78$	$11.65^{a}\pm0.42$
G1	$34.66^{abc}\pm1.08$	$25.60^{b}\pm1.02$	$192.40^{ab}\pm5.95$	$13.71^{a}\pm0.38$
G2	$45.80^{de}\pm0.62$	$33.32^{c}\pm1.23$	$205.00^{abc}\pm4.56$	$25.30^{bc}\pm1.11$
G3	$45.35^{de}\pm0.57$	$33.05^{c}\pm1.59$	$206.00^{abc}\pm8.18$	$26.32^{c}\pm1.80$
G4	$47.20^{de}\pm2.03$	$37.30^{d}\pm1.06$	$211.80^{bcd}\pm4.14$	$27.52^{cde}\pm1.00$
G5	$50.81^{f}\pm1.41$	$41.13^{ef}\pm1.21$	$222.80^{cde}\pm6.79$	$30.50^{def}\pm1.07$
G6	$48.15^{ef}\pm1.45$	$38.83^{de}\pm0.89$	$216.80^{cde}\pm8.55$	$28.29^{cde}\pm0.63$
G7	$48.92^{ef}\pm1.02$	$39.64^{def}\pm1.40$	$220.00^{cde}\pm3.97$	$28.68^{cde}\pm1.56$
G8	$51.32^{f}\pm1.40$	$42.50^{fg}\pm1.00$	$226.20^{de}\pm4.19$	$30.89^{ef}\pm0.37$
G9	$61.40^{g}\pm1.83$	$44.90^{g}\pm1.66$	$232.00^{e}\pm8.68$	$33.35^{f}\pm1.16$

G1: G9 refers to groups of rats that were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM.

As revealed from table 5, the administration of the combination of food additives produced elevations in liver functions (AST, ALT, ALP, and γ-GT) with different degrees in comparison to the control group. Rats of groups G5, G8, and G9 were almost the highest regarding liver functions. In the same context, the administration of food additives' combinations led to significant reductions in both total protein and albumin (Table 6), and significant elevations in total, direct, and indirect bilirubin (Table 6). G5, G8, and G9 groups seem to be inevitable to have the lowest levels of both total protein and albumin and the highest levels of bilirubin.

Although sucralose and acesulfame k are among safe artificial sweeteners, they adversely affect gut microbiota and cause an imbalance in gut microbiota (dysbiosis), increasing the emission of pro-inflammatory mediators in animal experiments, which results in an increase of inflammatory markers in the liver, such as MMP-2 and iNOS [57,58]. In the current study, the higher dose of these artificial sweeteners in food additives' combinations G1, G2, G3, G4, G7, G8, and G9 may interpret their negative effects on liver and kidney functions due to the dysbiosis, especially that [59] reported that dysbiosis is related to increase of pathogenic flora and enhanced permeability of the intestinal barrier which associated with increased inflammation and oxidative stress. The combination of all selected food additives in G9 made the worst impact on the biochemical parameters and the highest in terms of DNA damage, especially since there are studies that pointed to the harmful effect of each food additive individually, for example, but not limited the mentioned harmful effect of sucralose and acesulfame k, also tartrazine and carmoisine, organic azo dyes used in food products, drugs, and cosmetics, affect adversely liver at doses 8 and 100 mg/kg bw [52]. Additionally, monosodium glutamate persuades renal toxicity and oxidative stress [60]. Exposure to doses

around the acceptable daily intake of thirteen common chemicals, for one year, induced non-monotonic rises of AST and ALT in rats [61].

Table 6. Total protein, albumin, and bilirubin of rats were treated with different combinations of food additives.

Groups of rats (n=5)	T. Protein (g/dl)	Albumin (g/dl)	T. Bilirubin (mg/dl)	D. Bilirubin (mg/dl)	Ind. Bilirubin (mg/dl)
Control	$7.06^{h}\pm0.35$	$4.88^{i}\pm0.14$	$2.50^{ab}\pm0.13$	$1.68^{a}\pm0.11$	$0.83^{ab}\pm0.11$
G1	$6.46^{g}\pm0.23$	$4.52^{ghi}\pm0.17$	$2.46^{a}\pm0.14$	$1.75^{ab}\pm0.43$	$0.71^{ab}\pm0.11$
G2	$5.84^{ef}\pm1.14$	$4.35^{fgh}\pm0.18$	$3.04^{cde}\pm0.17$	$1.9^{abcd}\pm0.08$	$1.14^{abc}\pm0.16$
G3	$5.45^{de}\pm0.16$	$4.31^{fgh}\pm0.10$	$3.02^{cde}\pm0.08$	$1.88^{abcd}\pm0.13$	$1.14^{abc}\pm0.19$
G4	$5.07^{cd}\pm0.12$	$4.16^{efgh}\pm0.07$	$3.07^{cde}\pm0.14$	$1.95^{abcd}\pm0.06$	$1.12^{abc}\pm0.17$
G5	$4.08^{a}\pm0.06$	$3.09^{bc}\pm0.08$	$4.01^{f}\pm0.13$	$2.47^{efg}\pm0.15$	$1.54^{cd}\pm0.19$
G6	$4.27^{ab}\pm0.08$	$3.93^{de}\pm0.17$	$3.42^{e}\pm0.12$	$2.12^{bcde}\pm0.15$	$1.29^{bcd}\pm0.13$
G7	$4.11^{ab}\pm0.08$	$3.53^{cd}\pm0.21$	$3.43^{e}\pm0.12$	$2.24^{def}\pm0.10$	$1.20^{abc}\pm0.17$
G8	$3.95^{a}\pm0.15$	$3.05^{b}\pm0.24$	$4.10^{f}\pm0.16$	$2.53^{fg}\pm0.08$	$1.57^{cd}\pm0.14$
G9	$3.88^{a}\pm0.17$	$2.46^{a}\pm0.23$	$4.44^{f}\pm0.26$	$2.63^{g}\pm0.11$	$1.81^{d}\pm0.29$

G1: G9 refers to groups of rats that were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM

3.4. In vivo comet assay of food additives' combinations.

Regarding the results of comet assay in brain, kidney, and liver tissues (Table 7, 8, and 9 respectively), in summary, it was clear that the studied food additives' combinations exhibited genotoxic activities with different degrees compared to control rats in the comet assay in the brain, kidney, and liver.

The highest DNA damaged cells in the brain, kidney, and liver tissues were recorded for G6, G7, G5, G8, and G9 groups, respectively, rats of groups G5, G6, G8, and G9 recorded the highest numbers (7, 7, 7 and 11 respectively) of brain cells in comet class 3 (the longest tail). Rats of groups G5, G6, G7, G8, and G9 recorded the highest numbers (9, 10, 10, 14, and 16 respectively) of kidney cells in comet class 3 (the longest tail). Rats of groups G8, G7, G5, and G9 recorded the highest numbers (11, 12, 13, and 23 respectively) of liver cells in comet class 3 (the longest tail).

In the present study, *in vivo* comet assay pointed to DNA damage either in the brain, kidney, and liver cells. This DNA damage may be due to the release of reactive oxygen species. Reactive oxygen species attack amino acids and form carbonyl groups, which commonly indicate to protein oxidation. DNA-protein crosslinking (DPC) can occur when a protein becomes covalently bound to DNA and enhanced via elevated carbonyl content of proteins. DNA-protein crosslinking interferes with DNA replication, transcription, and repair, which might result in persistent DNA damage [62].

Table 7. Rate of DNA damage in brain tissues of rats were treated with different combinations of food additives using the comet assay.

Groups of rats (n=3)	No. of cells		Class of comet¥				DNA damaged cells (mean ± SEM)
	Analyzed*	Total comets	0	1	2	3	
Control	300	19	281	18	1	0	6.33 ± 0.58^{c}
G1	300	22	278	11	9	2	7.34 ± 0.55^{bc}
G2	300	23	277	16	5	2	7.67 ± 0.30^{bc}
G3	300	24	276	17	5	2	8.00 ± 0.48^{bc}
G4	300	28	272	19	6	3	9.33 ± 0.27^{b}
G5	300	43	257	22	14	7	14.31 ± 0.77^{ab}
G6	300	32	268	17	8	7	10.65 ± 0.75^{b}

Groups of rats (n=3)	No. of cells		Class of comet¥				DNA damaged cells (mean ± SEM)
	Analyzed*	Total comets	0	1	2	3	
G7	300	41	259	23	13	5	13.67±0.76^{ab}
G8	300	44	256	22	15	7	14.65±0.76^{ab}
G9	300	52	248	23	18	11	17.31±0.29^{a}

¥: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No. of cells analyzed were 100 per animal. G1: G9 refers to groups of rats were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM.

Table 8. Rate of DNA damage in kidney tissues of rats was treated with different combinations of food additives using the comet assay.

Groups of rats (n=3)	No. of cells		Class¥ of comet				DNA damaged cells (mean ± SEM)
	Analyzed*	Total comets	0	1	2	3	
Control	300	20	280	19	1	0	6.67±0.76^{d}
G1	300	23	277	11	10	2	7.67±0.75^{cd}
G2	300	26	274	17	5	4	8.67±0.77^{c}
G3	300	27	273	18	6	3	9.00±0.50^{c}
G4	300	33	267	21	8	4	11.00±0.52^{bc}
G5	300	50	250	25	16	9	16.67±0.75^{b}
G6	300	47	253	25	12	10	15.67±0.59^{b}
G7	300	48	252	20	18	10	16.00±0.88^{b}
G8	300	52	248	21	17	14	17.33±0.75^{ab}
G9	300	58	242	23	19	16	19.33±0.29^{a}

¥: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No. of cells analyzed were 100 per animal. G1: G9 refers to groups of rats that were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM.

The elevated rate of DNA damage in groups G5 and G9 may be related to the presence of sodium nitrite, which is supported by [51], who reported that sodium nitrite mediated genotoxicity and DNA damage. This effect of sodium nitrite may be either directly via chemical alteration by free radicals [63] or indirectly via elevated lipid oxidation products such as unsaturated aldehydes and MDA, which can link to DNA to produce mutagenic lesions [64]. Monopotassium glutamate and Magnesium diglutamate increased DNA damages observed by *in vitro* comet assay, and have clastogenic, mutagenic, aneugenic, and cytotoxic effects in human lymphocytes *in vitro* [65], carmoisine has a cytotoxic and genotoxic impact on meristematic cells of *Allium cepa* [66], Caramel colors are not genotoxic or carcinogenic, and exposure estimates designate that intake of caramel colors do not cause undue safety risks [67], although caramels could be contaminated with 2-acetyl-4-(1,2,3,4-tetrahydroxybutyl)imidazole (THI) and 4-methylimidazole (4-MEI), THI and 4-MEI are – to a certain level – allowed to be present in the food caramel colors, THI and 4-MEI might be hazardous to human health [68].

Table 9. Rate of DNA damage in liver tissues of rats was treated with different combinations of food additives using the comet assay.

Groups of rats (n=3)	No. of cells		Class¥ of comet				DNA damaged cells (mean ± SEM)
	Analyzed*	Total comets	0	1	2	3	
Control	300	20	280	17	3	0	6.65±0.76^{d}
G1	300	25	275	14	10	1	8.31±1.04^{cd}

Groups of rats (n=3)	No. of cells		Class¥ of comet				DNA damaged cells (mean ± SEM)
	Analyzed*	Total comets	0	1	2	3	
G2	300	29	271	21	5	3	9.67±0.57^{c}
G3	300	32	268	15	14	3	10.67±0.76^{c}
G4	300	36	264	23	9	4	12.00±0.50^{bc}
G5	300	58	242	24	21	13	19.33±0.75^{ab}
G6	300	45	255	21	16	8	15.00±0.50^{b}
G7	300	54	246	22	20	12	18.00±0.50^{ab}
G8	300	59	241	27	21	11	19.67±0.75^{ab}
G9	300	65	235	23	19	23	21.66±0.76^{a}

¥: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus. (*): No. of cells analyzed were 100 per animal. G1: G9 refers to groups of rats that were orally administrated mixtures of food additives, as shown in table (1). Columns superscript with different letters are significantly different ($p < 0.05$). The data are expressed as mean values ±SEM.

Human has daily exposure to many types of chemicals, such as different types of preservatives from cosmetics, pesticides from fruits/vegetables, food additives, antibiotics, and other veterinary drugs from food of animal origin, so classical animal studies designed to test the toxic outcome of a single chemical are not suitable to assess the effects of the whole mixture of chemicals human have daily contact with it [69]. Cell proliferation, which plays a key role in fixing mutations induced by DNA damage, may occur as a result of the combined effects of chemicals in food and enhances of the mutagenic effect of mixture [70].

4. Conclusions

Although studied food additives have NOAELs in literature and are expected to be relatively safe laboratory animals, the combinations of some food additives at their NOAELs, according to this study, seem to be unsafe as it should be; These combinations made a worth impact on the biochemical parameters and exhibited genotoxic activities with different degrees compared to control rats in the comet assay in the brain, kidney, and liver.

Further studies on food additives' combinations are required regarding additive-additive interactions, additive-food interactions, and the effect of processing conditions on these food additives.

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Conflicts of Interest

The authors declare no conflict of interest.

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