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Adverse Effects of Two Kinds of Food Additives Mixtures (Fast green + Glycine, Fast green+ Sodium Nitrate and Sodium Nitrate + Glycine) on Some Physiological Parameters in Male Albino Rats

Asmaa A. H. Mohamed[1], Eman G.E. Helal*[1], Mohamed A. Abdelaziz[2]
[1]Department of Zoology, Faculty of Science (Girls), Al-Azhar University, Egypt,
[2]Physiology Department, Faculty of Medicine, (Boys), Al –Azhar University, Egypt
***Corresponding Author**: Eman Helal, **Email**: emanhelal@hotmail.com,
Mobile: 00201001025364, orcid.org/0000-0003-0527-7028

ABSTRACT
Background: Food additives are substances used in food industry in order to improve the food's taste and appearance. **Objective:** To investigate the adverse effects of mixing some food additives on the biochemical parameters in male albino rats. **Materials and Methods:** This study was conducted on twenty-four young male albino rats. Animals were divided into four groups (6 each). **Group I:** Control group**, Group II:** administered with fast green and glycine. **Group III:** administered with fast green and sodium nitrate and **Group IV:** administered with sodium nitrate and glycine. After 30 days of treatment, blood samples were collected for biochemical parameters.
Results: body weight, total protein, albumin and testosterone hormone decreased in all treated groups. Glucose, insulin and HOMA-IR decreased in group received (fast green with glycine). Rats treated with sodium nitrate plus glycine showed a significant increase in glucose level. The liver enzymes and the kidney function tests increased in all treated groups. Rats treated with fast green plus glycine showed significant decrease in the TG and TC while HDL showed significant increase. Rats treated with sodium nitrate plus glycine showed a significant increase in the TG and TC while HDL showed significant decrease. T3 and T4 decreased in rats treated with the mixture of sodium nitrate and glycine while increased in rats treated with the mixture of fast green and glycine.
Conclusion: The use of these compounds must be limited as its use leads to real vehement disturbance in biochemical parameter.
Keywords: Biochemical parameter, Food additives, Sodium nitrate, Thyroid hormones.

INTRODUCTION
Food additives are substances that are added to food to preserve its flavor or improve its taste, appearance, or other qualities. Food additives are substances with little or no nutritional value, but are used in the processing or storage of foods or animal foods, especially in developed countries. Antioxidants include food preservatives, food colouring agents flavouring agents, anti-infective compounds, excipients and other similar substances **(1)**. Food preservatives are additives that are used to prevent the growth of bacteria, molds, and yeasts in food **(2)**.

Fast green (FG) is a bluish green food dye that provides a dark green shade in applications (3). Recently, the potential role of food additives in food intolerance has received particular attention, and FG was showed to have toxicity, modifying the immediate allergic response when ingested orally in food **(4)**, impairing hepatic functions **(5)** and inhibiting synaptic activity in rat hippocampal interneurons **(6)**.

Glycine is a colorless, sweet-tasting crystalline solid. Glycine is used with food as flavouring. It is mildly sweet, and it counters the aftertaste of saccharine. It also has preservative properties, perhaps owing to its complexation to metal ions **(7)**.

Sodium nitrate ($NaNO_3$) is a food preservative used for meat. Nitrates are used in curing, which is a broad category of techniques for preserving foods, mainly meat and fish that involves the use of salt, sugar, or some form of dehydration. In each case, the goal is to make the food unattractive to the bacteria that cause food spoilage. Bacteria are tiny organisms that require, among other things, moisture, oxygen and food. Taking away one of these things, they die. One special property of sodium nitrate is that it prevents the growth of Clostridium botulinum **(8)**. Sodium nitrate can be dangerous to human health because it causes methemoglobinemia and because it is associated with carcinogenic effects. Sodium nitrate ($NaNO_3$) does not have any negative effects on our body, but when we eat sodium nitrate, a portion of it, about 5%, is converted into sodium nitrite ($NaNO_2$) by the bacterial flora in the oral cavity. Sodium nitrite is the real dangerous molecule that causes methemoglobinemia in the blood and has carcinogenic effects **(9)**.

This work aimed to investigate the adverse effects of mixing some food additives on the biochemical parameters in male albino rats.

MATERIALS AND METHODS
Twenty-four young male albino rats (weighing 120-140 g) were used in this study. Animals were housed in stainless steel cages, fed on rat chow and offered water *ad libitum*. The animals were divided into four groups. The first group (the control) untreated group, the second group was orally administered fast green (12.5 mg/kg b.w./day) and glycine (15 mg/kg b.w./day), the third group was orally administrated with fast green (12.5

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mg/kg b.w./day) and sodium nitrate (10 mg/kg b.w./day) and the fourth group was orally administrated sodium nitrate (10 mg/kg b.w./day) and glycine (15 mg/kg b.w./day). After 30 days of treatment, animals were weighed then decapitated. Blood samples were collected for biochemical parameters. Blood samples were centrifuged for 10 min at 5000 rpm and supernatant sera were separated for analysis without storage or delay.

Biochemical Examination:

In the present study, total protein (TP) and albumin concentration were estimated, and then serum globulin concentrations were calculated according to the formula: Globulin (g/dl) = total protein (g/dl) – albumin (g/dl).

Aspartate aminotransferase (ASAT) and alanine aminotransferase (ALAT), creatinine, urea, glucose concentrations as well as lipid profile (including total cholesterol, triglycerides, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol) were also determined.

Concentrations of testosterone and thyroid hormones (T3 and T4) were measured. All parameters were estimated (using Liaison from Diasorin Italy SA kits, France). However, ratios of TC/HDL (risk ratio 1) and LDL/HDL (risk ratio 2) were also calculated after calculation of serum LDL-C (low-density lipoprotein cholesterol) and VLDL (very low density lipoprotein cholesterol) using the Friedewald's [10] and Norbert [11] formulas, respectively as follows: Friedewald's [10] equation: LDL (mg/dl) = TC- {HDL + [TG/5]}. Norbert [11] equation: VLDL = TG/5.

Determination of serum insulin level and HOMA-IR:

By using an ELISA (Enzyme Linked Immunosorbent Assay) kit (U.E Type) for measurement of rat insulin with high sensitivity and rapidly using (Biovendor Research and Diagnostic Product ref.). The approximating equation for insulin resistance, in the early model used fasting plasma sample, and was derived by use of the insulin-glucose product, divided by a constant: HOMA –IR = Glucose mg/dl x Insulin µu/L/405. Fasting glucose in mass units mg/dl. IR is insulin resistance. Insulin is given in µu/L [12] .

Ethical approval:

This study was conducted in accordance with ethical procedures and policies approved by Animal Care and Use Committee of Faculty of Science, Al-Azhar University, Cairo, Egypt. The study was approved by the Ethics Board of Al-Azhar University.

Statistical analysis

The results were expressed as mean ± SE of the mean. Data were analyzed using the Statistical Package for the Social Sciences (SPSS) program, version 25. P less than 0.05 was considered significant.

RESULTS

- % of Body weight change and glucose level:

The percent change in the body weight showed highly significant decrease in all treated group. On the other hand, glucose level, insulin and HOMA-IR revealed a highly significant increase in the group that received sodium nitrate with glycine and insignificant changes in the group that treated by sodium nitrate and fast green. While, rats treated with fast green and glycine showed significant decrease in glucose, insulin and HOMA-IR levels as compared to the control (Table 1).

Table (1): The effect of food additives on body weight change (BWC), glucose, insulin hormone and HOMA-IR

Group	Control	Fast green + glycine	Fast green + sodium nitrate	Sodium nitrate + glycine
BW (g)	35.78±0.59	31.74±0.55**	27.10±0.74**	31.00±0.70**
% of change		-11.29 %	-24.26 %	-13.36 %
Glucose (mg/dl)	75.40± 0.70	71.42±0.54**	77.20±1.06	83.50±0.70**
% of change		-5.28 %	2.39 %	10.74 %
Insulin (ng/dl)	4.04± 0.06	3.80±0.07**	4.09±0.05	5.26±0.09**
% of change		-5.94 %	1.24 %	30.20 %
HOMA-IR (ng/dl)	0.74± 0.01	0.67±0.01*	0.77±0.01	1.09±0.02**
% of change		-9.46 %	4.05 %	47.30 %

Values represent mean ± SE (standard error). P*<0.05, P**<0.01as compared to control group.

- The results of Protein profile:

Our results showed significant decrease in serum total protein level in all treated groups, while albumin level showed significant decrease in the groups treated by fast green and sodium nitrate, and the group treated with fast green and glycine as compared to control group. The globulin level showed highly significant decrease in the groups treated by mixture of fast green + glycine and fast green + sodium nitrate and showed significant decrease in the group of rats that was treated by mixture of sodium nitrate + glycine. The A/G ratio showed highly significant increase in the groups of rats that were treated by mixture of fast green + glycine and fast green + sodium nitrate and showed significant increase in the group that was treated by mixture of sodium nitrate + glycine.

Table (2): The effect of food additives on total protein, albumin, globulin and albumin/globulin ratio

Group	Control	Fast green + Glycine	Fast green + sodium nitrate	Sodium nitrate + Glycine
Total protein (g/dl)	6.18±0.08	5.27±0.18**	4.10±0.07**	5.89±0.05*
% of change		-14.72 %	-33.66 %	-4.69 %
Albumin (g/dl)	3.44±0.03	3.12±0.12*	2.88±0.05**	3.49±0.08
% of change		-9.30 %	-16.28 %	1.45 %
Globulin (g/dl)	2.74±0.11	1.92±0.11**	1.22±0.09**	2.41±0.05*
% of change		-29.93 %	-55.47 %	-12.04 %
Albumin/Globulin	1.26±0.06	1.66±0.17**	2.39±0.16**	1.48±0.04*
% of change		31.75 %	89.68 %	17.46 %

Values represent mean ± SE (standard error). P*<0.05, P**<0.01 as compared to control group.

Liver functions:

Our results showed that there was significant increase in the activities of the ASAT and ALAT enzymes in all rat groups treated by different food additives combinations for 30 days as compared to control rats (Tables 3).

Table (3): The effect of food additives on serum alanine transaminase and aspartic transaminase

Group	Control	Fast green + glycine	Fast green + sodium nitrate	Sodium nitrate + glycine
ALAT (IU/l)	22.98±0.47	38.32±0.98**	43.38±0.75**	35.80±0.86**
% of change		66.75 %	88.77 %	55.79 %
ASAT (IU/l)	51.71±0.31	64.00±1.30**	62.54±0.81**	62.80±0.86**
% of change		23.77 %	20.94 %	21.45 %

Values represent mean ± SE (standard error). P*<0.05, P** <0.01as compared to control group.

Kidney functions:

The recorded results of renal function parameters including (urea and creatinine) showed a significant increase in all food additives combinations-treated groups in comparison with the control group (Table 4).

Table (4): The effect of food additives on serum creatinine and urea

Group	Control	Fast green + Glycine	Fast green + sodium nitrate	Sodium nitrate + Glycine
Creatinine (mg/dl)	0.91±0.05	1.48±0.13**	1.36±0.15**	1.60±0.09**
% of change		62.64 %	49.45 %	75.82 %
Urea (mg/dl)	30.34±0.53	40.00±0.70**	41.14±0.63**	41.80±0.86**
% of change		31.84 %	35.60 %	37.77 %

Values represent mean ± SE (standard error). P*<0.05, P** <0.01as compared to control group

Lipid profile:

Present results showed significant increase in TC, TG, LDL, VLDL and ratios of TC/HDL (risk ratio 1) and LDL/HDL (risk ratio 2) accompanied with marked decline in HDL in sodium nitrate plus glycine-treated group as compared to the control group. In fast green plus glycine-treated group, there was a significant decrease in TC, TG, LDL, VLDL and ratios of TC/HDL (risk ratio 1) and LDL/HDL (risk ratio 2) accompanied with marked increase in HDL as compared to the control group. In the group treated by fast green plus sodium nitrate, there was an insignificant change as compared to the control group (Table 5).

Table (5): The effect of food additives on serum cholesterol (TC), triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL) and LDL/HDL and TC/HDL ratios

Group	Control	FG + GL	FG + SN	SN + GL
Cholesterol (mg/dl)	80.00±1.14	77.00±0.70*	80.60±.92	94.80±1.49**
% of change		-3.75 %	0.75 %	18.50 %
Triglycerides (mg/dl)	75.78±0.36	72.00±0.70**	76.30±1.09	87.40±0.92**
% of change		-4.99 %	0.69 %	15.33 %
HDL (mg/dl)	43.75±0.53	47.60±1.02**	40.60±1.36	34.40±1.80**
% of change		8.80 %	-7.20 %	-21.37 %
LDL (mg/dl)	21.09±0.78	15.00±1.46**	24.58±1.98	42.92±2.80**
% of change		-28.88 %	16.55 %	103.51 %
VLDL (mg/dl)	15.15±0.07	14.40±0.14**	15.42±0.29	17.48±0.18**
% of change		-4.95 %	1.78 %	15.38 %
LDL/HDL	0.48±0.01	0.31±0.03**	0.60±0.07	1.27±0.13**
% of change		-35.42 %	25.00 %	164.58 %
TC/HDL	1.82±0.01	1.61±0.04**	1.99±0.08	2.57±0.16**
% of change		-11.54 %	9.34 %	41.21 %

Values represent mean ± SE (standard error). P* <0.05, P** <0.01as compared to control group.

Hormones:

The thyroid hormones T3 and T4 were significantly decreased in the group treated by mixture of sodium nitrate + glycine, while significantly increased in the group treated by the mixture of fast green + glycine and showed insignificant changes in the group treated by the mixture of fast green + sodium nitrate. The testosterone hormone was decreased significantly in all treated groups.

Table (6): The effect of food additives on serum tri-iodothyronine (T_3), thyroxine (T_4) and testosterone hormones

Group	Control	Fast green + Glycine	Fast green + sodium nitrate	Sodium nitrate + glycine
T_3 hormone (ng/dl)	108.22± 0.83	116.40±1.20**	109.70±0.53	93.20±1.15**
% of change		7.56 %	1.37 %	-13.88 %
T_4 hormone (ng/dl)	4.57±0.15	5.92±0.08**	4.13±0.05*	3.49±0.17**
% of change		29.54 %	-9.63 %	-23.63 %
Testosterone hormone (ng/dl)	57.30±0.42	42.20±0.86**	41.00±0.70**	41.20±0.86**
% of change		-26.35 %	-28.45 %	-28.10 %

Values represent mean ± SE (standard error). P*<0.05, P**<0.01 as compared to control group.

DISCUSSION

In the present work, all treated groups showed significant decrease in the body weight. These results agree with the previous study by **Abou El-Zahab *et al.*** [(13)] who stated that any synthetic food coloring agent cause decrease in the body weight as the fast green substance. Nitrate could induce body weight loss by the way of inhibiting food intake through its influence on the nervous regulation of feeding behavior, that was found to cause a decrease of growth hormone receptors in the liver, thus causing lack of plasma somatomedins [(14)], which in turn affect body growth. The glycine has insignificant change on the body weight. In the present study, the glucose level decreased in rats that were treated by the mixture of fast green plus glycine. The fast green caused decrease in glucose level, which may be attributed to impairment of hepatic functions [(15)]. Fast green may indirectly play a specific role in carbohydrate metabolism. Our results clearly showed that rats treated with the mixture of sodium nitrate plus glycine showed significant increase in serum glucose, insulin and insulin resistance (HOMA-IR) concentration. Nitrate-treated animals displayed improved hyperglycemia, improved insulin sensitivity and glucose tolerance, elevated pancreatic islet content, insulin content and insulin secretion. In addition, improved inflammation, dyslipidemia and liver function. **Shelpov *et al.*** [(16)] reported that in the presence of nitrate ions, the activity of amylase and phosphorylase increased leading to the liberation of glucose from glycogen, so blood glucose increased while liver glycogen decreased. Other findings suggested stimulation by nitrate of gluconeogenesis and glucose shift from tissue to blood or an impairment of glucose mobilization [(17)]. We can expect that nitrate produces hyperglycemia due to a deficiency of insulin release. Nitrate opens potassium channels, which through closing voltage-gated calcium channels decreases intracellular calcium. Calcium is known to trigger insulin secretion and calcium channel blockers are known to produce hyperglycemia [(18)]. The antagonistic effect of fast green and sodium nitrate cause insignificant change in glucose and insulin levels in rats treated by the mixture of sodium nitrate plus fast green. Our results stated that there was significant decreased in the level of total protein in all rats treated by different mixtures. Fast green caused an increase in amino acids deamination as a result of the presence of some toxic compounds [(19)]. The decrease was due to the reduction of serum globulin level, which showed marked decline at the same time. The decrease may be an indication of the delayed depression effect of colorant used on immunoglobulin production, the defense mechanism that aims to protect the body from toxic effects. The decrease in total protein concentration may result from nitrate toxicity mediated through formation of nitric oxide or peroxynitrite, which oxidizes proteins and lipoproteins. It is clear that nitrate decreases total serum protein mainly through its effects on the liver, either through the necrotic changes, especially of the plasma membrane or through the inhibition of the oxidative phosphorylation process at first and then the availability of energy source for protein synthesis and other metabolic processes [20].

Our results stated that the liver enzymes (ALAT and ASAT) increased in all treated rats. This was due to the toxic agent mainly affecting the liver functions. The observed elevation in serum ASAT and ALAT activities in response to the administration of fast green + glycine is in agreement with the study of **Mekkaway *et al.***[21] who found that activities of ASAT and ALAT were increased significantly following fast green treatment to rats. This may be due to hepatic potency of the fast green substance resulting in destructive changes in the hepatic cell. The liver enzymes are normally found in circulation in small amounts because of hepatic growth and repair. Earlier findings reported the increase in the level of ASAT and ALAT in sodium nitrate-treated rats due to the formation of free radical ONOO- from nitric oxide. Both NO and oxygen radicals could react further to produce other oxidants and nitro compounds such as peroxynitrite to induce liver injury and to play an important role in death of liver cells [22].

Our result stated that the kidney function markers (urea and creatinine) were increased in all treated groups. This because fast green enhanced protein catabolism and accelerated amino acid deamination for gluconeogenesis, which is possibly an acceptable postulate to interpret the elevated levels of Urea [23]. The increments in uric acid concentrations might be due to degradation of purines or to an increase of uric acid levels by either over production or inability of excretion [24]. The increment in creatinine concentration in response to fast green intake agrees with that observed by **Mekkawy *et al.*** [21]. Elevated creatinine concentration is associated with abnormal renal function, especially as it relates to glomerular function. Our result stated that there were high levels of urea and creatinine in serum of nitrate-exposed rats, however an increased urinary protein loss was also found. This protein loss probably resulting from nitrate-induced kidney dysfunction [25].

Our results demonstrated that triglycerides and total cholesterol levels were decreased in response to fast green and glycine oral administration to the rats. These results are in agreement with **Ashour and Abdelaziz** [26] who noticed a significant reduction in serum total cholesterol and triglycerides level when food color azo dye (fast green) was consumed orally to male albino rats for 35 days . The possible explanation of these observed decrements may reside direct and indirect action of fast green on lipid metabolism or lipid peroxidation [26].

Our results also demonstrated that there was increase in lipids in rats treated by the mixture of sodium nitrate plus glycine. In this regard, a number of investigations have been focused on the influence of nitrate on the thyroid status. Some workers showed thyroid hypertrophy with decreased thyroid hormone levels in people who consume drinking water with nitrate concentrations below or above WHO nitrate standard. According to **Luboshitzky *et al.*** [27] sub-clinical hypothyroidism characterized by a decreased T4 and an increased TSH concentration was associated with elevated total cholesterol and LDL-C concentrations. Also, serum triglyceride concentrations were significantly elevated, which could be related to a reduced removal rate of triglycerides from plasma in case of hypothyroidism [28].

Because the antagonistic effect of sodium nitrate and fast green there was insignificant change in lipids on rats treated by the mixture of fast green and sodium nitrate. In our work, there was decrease in thyroid hormones in rats treated by the mixture of sodium nitrate plus glycine. Sodium nitrate decreased the thyroid hormone T3 and T4 because nitrate acts as a dose-dependent competitive inhibitor of sodium iodide symporter (NIS) that mediates the uptake of iodine by the thyroid. Sufficiently decreased iodine uptake by the thyroid may result in decreased production of thyroid hormones T3 and T4. Sufficiently inhibited uptake of iodine by the thyroid could result in effects associated with thyroid dysfunction (e.g., hypothyroidism). Concerning nitrate-induced effects on thyroid function, it prompted scientists to perform studies designed to assess thyroid function relative to drinking water and/or dietary nitrate levels [29].

The increase in T3 in rats treated by mixture of fast green plus glycine could be a result of peripheral conversion of T4 to T3 which increased the parenchymal cells of the liver because of the presence of reactive oxygen species during the biotransformation of the dye. Testosterone hormone decreased in all treated groups. The reduced cellularity of the interstitium in the testes of the rats treated by nitrate might produce a decrease in testosterone and consequently poor spermatogenesis [30]. Our findings showed that fast green have many harmful effects on spermatogenesis and steroidogenesis in rats. It is a fact that testosterone is an essential factor controlling testicular development and sperm apoptosis, a lower concentration of this indispensable androgen is usually associated to a decrease in Leydig cells activity or to a lower testosterone capture in testis [31].

Our results demonstrated that serum testosterone level was reduced in rats treated by glycine. The central nervous system of glycine-treated rats showed neurogenic functional changes in the hypothalamus that induced a reduction in levels of LH, FSH and testosterone. It was reported that glycine destroyed neurons of the hypothalamus in rats. Such neuronal losses in the hypothalamus can result in disruption of the hypothalamic-pituitary-testis regulatory axis that controls the steroidogenesis of testicular Leydig cells [32]. This will lead to decrease of serum testosterone levels recorded in the present work due to presence of 5'deiodinase because of morphological changes**.**

CONCLUSION

The use of these compounds must be limited as its use leads to real vehement disturbance in biochemical parameters.

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