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THE POTENTIAL ADVERSE EFFECTS OF SUB-CHRONIC ADMINISTRATION OF SOME FOOD COLORING ADDITIVES ON YOUNG MALE ALBINO RATS.

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ABSTRACT

Different food coloring additives (**New Coccin, Sunset yellow** and **fast green dyes**) are commonly used, were administered to healthy young male albino rats separately or in combination for 6 weeks period to evaluate their effects on liver and kidney functions, hepatic lipid peroxidation, protein carbonyl, hydroxyl proline and glutathione contents as well as serum zinc and iron concentrations. Results showed significant increases in urea, uric acid, creatinine and bilirubin levels in serum of food colorants administered rats either separately or in combined administration. In addition, significant elevations in the activities of serum ALT, AST, LDH and γ-GT as well as in the hepatic hydroxy proline content were recorded. The results also revealed depletion in the hepatic reduced glutathione (GSH) content in contrast to significant increases in malondialdehyde (MDA), a product of lipid peroxidation (TBARS) and carbonyl protein (CP) contents. The results, further more, showed significant depletion in serum zinc concentration but significant increase in iron concentration. Regarding serum protein fractions, food colors caused a slight changes except fast green and combined treatment which revealed remarkable increase in albumin and globulin fractions specially gamma globulin. However, there were no remarkable changes in the treated rats general appearance except hyperactivity particularly in the animal group taken the combined colors. Meanwhile, no death occurred in any experimental group.

In conclusion, the results of the present study indicated that, ingestion of food color additives caused serious health effects in young male rats .It can be recommended that, all non-essential food additives such as colorants should be banned. At least in order to protect the health of young children, youths, adolescents and adults, generally of our future generation.

Key words: Food colour additives, hepatic and renal functions, lipid and protein oxidation, zinc and iron.

INTRODUCTION

Food additives are widely used for technological recenthy, in Egypt purposes and their presence is often substantial daily diet **[Stefanidou et al., (2003)]**. In Egypt, there is a sharp increase in the use of synthetic food colorants in the last few years **[Saleh (1994)]**. The association of food additives with hyperactivity is a popularly accepted notion. **Feingold (1975)** hypothesized that food dyes are pharmacologically active substances that induce or aggravate symptoms of hyperactivity in children. Subsequent studies have confirmed that food colors can induce clinical symptoms of hyperactivity **[Boris & Mandel (1994) and Bateman et al., (2004)]** and can also alter brain electrical activity in children **[Uhlig et al., (1997)]**.

Administration of non-nutritional food additive during critical development has been implicated in the induction and severity of some child hood behavioral and developmental disorders, such as attention deficit hyperactivity **[Boris & Mandel, (1994)]**. Also, controlled clinical trials that have eliminated food colorings from the diets of hyperactive children have shown an improvement in behaviour rating and health **[Bateman et al., (2004)]**. On the other hand, assessing the combined effect of chemicals is extremely complex. These effects include: a. additivity, where agents are no more and no less effective in combination or separately b. **[Axelrad et al., (2002)]**.

Synthetic dyes, such as new coccin red color, sunset yellow and fast green are widely used as food colors in many countries. Epidemiological studies of food color additives are difficult, because exposure cannot be accurately assessed. Thus, risk assessment largely depends on laboratory toxicity studies.

New coccin red dye, sunset yellow dye and fast green dye are azo dyes. Many azo dyes are genotoxic in short-term tests and carcinogenic in laboratory animals **[Combes & Haveland-Smith, (1982)]**. The only published report on their toxicity were performed on adult male rats .New coccine, which is not permitted as a food color in the US. A long-term toxicity study of New coccine in rats exposed in utero showed no carcinogenicity **[Brantom et al., (1987)]**. Water-soluble azo dyes such as new coccine have highly charged sulfonate

groups that preclude significant absorption of ingested dye. When the intact dye reaches the intestine, it can undergo extensive metabolic reduction by intestinal micro flora **[Levine (1991)]**, and the reductive cleavage products are rapidly absorbed **[Chung et al., (1992)].** It is also possible for mammalian azoreductase in the intestinal wall or liver to reduce the dye to free aromatic amines **[Chung & Cerniglia (1992)]**. In recent years, attention has been focused on the role of biotransformation of chemicals to highly reactive metabolites that initiate cellular toxicity. Many compounds, including clinically useful drugs, can cause cellular damage through its metabolic activation to highly reactive compounds. This study aims to evaluate the physiological consequences of ingesting three common favorable food color additives (used in a wide scale) new coccin red color, sunset yellow and fast green dyes, administered individually or in combination to young male albino rats.

MATERIALS AND METHODS.

Experimental animals:

Young male albino rats weighing 65-75 g (21-28 days old) were used in this experiment, obtained from Vaccine and Serum Institute Animal House, were held for 3 days before the beginning of the experiment.

The animals were maintained under normal condition, fed standard chow and water *ad libitum*.All experiments were carried out in accordance with protocols approved by the local experimental animal ethics committee.

The food color additive used in the experiment.

Commercially available food color additives that widely spread throughout the egyptian food market were used, particularly in Demietta County. New coccin (red color) (C.I.14720) banned in Sweden, USA, Austria and Norway, fast green FCF (C.I. 42053, certifiable as FD&C Green No. 3)- banned in Sweden, USA and Norway, Sunset Yellow FCF (C.I. 15985) banned in Norway.(50 mg of each color was dissolved alone or mixed up within 10 ml tap water.)Color solutions were administered in the drinking water at a concentration of 50mg /10ml/day continuously for a period of 6 weeks. The solution of color was freshly prepared daily and preserved in brown bottles covered with dark foil at room temperature.

EXPERIMENTAL PROTOCOL

The rats were divided into five groups of six animals each as follows.

1- Control untreated group, rats were fed a normal diet and drank tap water.

2- Color solution (I) group, animals administered new coccin (red color) daily with drinking water at a dose of 50 mg/ kg bw for six weeks.

3- Color solution **(II)** group, animals administered fast green dye, daily with drinking water at a dose of 50 mg/ kg bw for six weeks according to, [**Neuman et al., (1978) and Sasaki et al., (2002)].**

4- Color solution **(III)** group, animals administered sun set yellow color, daily with drinking water at a dose of 50 mg/ kg BW for six weeks.

5- Mixed color solution group, animal administered colored solution containing the three colors **(I, II&III),** by the same manner and the same dose (50/3 mg of each color) daily with drinking water for six weeks.

Biochemical assays.

At the end of the experimental duration (6 weeks), following overnight fasting, the animals were sacrificed by quick decapitation, blood samples were collected and the sera were separated and used for the biochemical assays. Further, liver samples were removed cleaned and weight immediately. 10% homogenates were prepared in sodium phosphate buffer (pH 7.0) .The following metabolic parameters were analyzed in the serum and liver homogenates.

Enzymatic activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were estimated by the methods of **[Reitman and Frankle (1957)].** Gamma- glutamyl transpeptidase was estimated by the method of **[Meister et al., (1981)].** While, lactic dehydrogenase (LDH) activity was determined according to **[Raabo (1963)].** Serum bilirubin and creatinine levels were estimated according to **[Routh (1976) and Faulkne & King (1976)]** respectively. Moreover, uric acid and urea were estimated in serum using the methods of **[Caraway (1955) and Haury (1965)],** respectively. The product of lipid peroxidation molondialdehyde (MDA) was determined in liver homogenates as described by **[Ohkawa et al. (1982)],** protein carbonyl (PC) content was estimated by the method of **[Smith et al. and expressed as µ Mol12, 4DNPH / mg (1991)]** while glutathione (GSH)

content was estimated by the method of **[Buetler et al. (1963)]**. Liver-hydroxy praline (HPRO) content was estimated by the method of **[Berg (1982)]**. The concentrations of zinc (Zn)and iron (Fe) were determined in serum after digestion in concentrated nitric acid by atomic absorption spectrophotometer with an air acetylene flame according to **[Zettner & Seligson (1964)]**. Electrophoresis was done according to the method described by **[laemmli (1970)]**. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.(SDS-PAGE) was performed using 70% slab gel. Protein bands in gel were stained with 0.1% coomassie brilliant blue R and destained in 10% acetic acid and 40% ethanol. The bands were identified and analyzed by gel protein analyzer. **Statistical Analysis:** All results were described as means ± standard error (SE). Significance of the changes between means was tested by the Student t-test at $P < 0.05$.

RESULTS

Serum enzyme activities.

The present study revealed that the administration of food color additives (New coccin (red color) (C.I.14720), fast green FCF (C.I. 42053, and sunset yellow FCF (C.i. 15985)-either individually or in combination to young rats caused a variable degrees of stimulation for their functions as reflected by the increased level of metabolic parameters. As shown in Table(1), serum enzyme activities of γ-GT, LDH, ALT and AST increased significantly after six weeks of colors administration either individually or in combination in which the most observed increase present in the combined treatment, if compared with control untreated group.

Kidney function tests.

Table (2): showed that the serum urea, uric acid, creatinine and total bilirubin levels increased significantly after six weeks of daily administration of the three color additives either given individually or in combination. The most observed elevations were seen in case of the combined treatment in comparison with the untreated control.

Table (1): Serum enzyme (GGT, LDH, ALT and AST) activities in different treated groups.

	Control	New Coccin Red color	Fast green color	Sun set Yellow color	Colors in combination
GGT(U/L)	214±1.59	379±7.57* +77.0	380.0±4.6* +78.0	351±5.39* +64.0	410±4.99* +92.0
LDH(U/L)	1682±82.6	2555±34.9* +52.0	1791±75.4 +6.5	2112±28.1* +25.56	3633±93.5* +116.0
AST(U/L)	41.4±2.46	76.6±2.73* +85.0	88.6±4.49* +114	74.8±3.62* +81.0	113.4±5.33* +174.0
ALT (U/L)	30.±2.1	45.2±0.37* +50.67	35.8±1.2* +19.33	36.0±1.7* +20	45.8±1.59* +52.67

*Significant as compared to control P<0.005.

Table (2): Serum Urea, Uric acid. Creatinine and Total Bilirubin levels in different treated groups.

	Control	New Coccin Red color	Fast green color	Sun set Yellow color	Colors in combination
Urea mg/dl	26.14±1.2	31.4±0.3* +20.2	31.9±1.24* +22.15	36.08±1.7* +38.03	37.61±1.4* +43.88
Uric acid mg/dl	2.85±0.08	3.29±0.16* +15.44	2.98±0.29 +4.56	3.05±0.07* +7.02	3.39±0.17* +18.95
Creatinine mg/dl	1.21±0.03	1.42±0.1* +17.36	1.76±0.1* +45.45	1.81±0.1* +49.59	2.12±0.23* 75.21
Total Bilirubin mg/dl	0.27±.009	0.32±0.01* +18.52	0.29±.009 +7.41	0.28±0.01 +3.7	0.35±0.01* +29.63

*Significant as compared to control P<0.005.

Lipid peroxidation, protein carbonyl and reduced glutathione status as well as hydroxy proline content.

The results of the present study (Table 3) revealed that food colorants treatment induced a hepatic disturbance of oxidative /antioxidative balance under investigation.

The data showed depletion in the reduced glutathione (GSH) content. In addition, there was a significant increase in lipid peroxidation (MDA) and carbonyl protein (PC) contents (marker of protein oxidation by the reactive oxygen species (ROS).as well as hydroxy proline content

after six weeks of administration each of the three colors. Combined color showed the most observed increase. as compared with control untreated young rats .

Serum Zinc and iron concentration.

The results of the present study. as shown in Table 4, observed that food colorants treatment given individually or in combination induced a significant increase in serum iron: meanwhile the serum zinc concentration exhibited a significant reduction comparing the normal group.

Serum Protein Electrophoresis.

Comparing with the normal group. electrophoretic data showed that. albumin and globulin fractions revealed remarkable elevations only in rats administered fast green food color as well as the combined colors. Concerning the other food colors administered to young rats. no appreciable changes have been recorded throughout the experimental period. **(Fig. 1a&1b)**

Table (3): Liver Lipid Peroxidation (MDA). Reduced Glutathione (GSH). Hydroxy Proline (HPRO) and Protein Carbonyl (PC) in different treated animal groups.

	Control	New Coccin Red color	Fast green color	Sun set Yellow color	Colors in combination
MDA n mol/g Fresh tissue	142±3.57	240±0.71* +69.0	209±6.23* +47.18	171.0±4.62* +20.42	266±5.78* +87.32
GSH mg/g Fresh tissue	0.17±0.01	0.08±0.005* -52.94	0.09±.002* -47.06	0.06±0.004* -64.71	0.05±0.004* -70.59
H PRO mg/g Fresh tissue	37.54±1.2	53.2±2.64 +41.72	44.08±2.61 +17.42	53.86±3.98 +43.47	56.58±3.35* +50.77
PCμMol2,4 DNPH/mg Fresh tissue	437±12.4	575±20.18* +31.58	557±14.7* +27.46	634±38.55* +45.08	866±24.57* +98.17

*Significant as compared to control P<0.005.

Table (4): Serum Iron and Zinc concentration in different treated animal groups.

	Control	New Coccin Red color	Fast green color	Sun set Yellow color	Colors in combination
Iron(Fe) µg/ml	35.5±2.15	39±2.32 +9.86	36.36=.62 +2.42	39.63±.83 +11.63	42=1.16* +18.31
Zinc(Zn) µg/ml	16=1.08	15.88±.83 -0.75	15.41=.35 -3.69	14.44±.24 -9 75	10.9±.47* -32.0

* Significant as compared to control (P<0.005).

DISCUSSION

Administration of non-nutritional food additive during critical development has been implicated in the induction of some cellular disorders. The present data showed that the administration of the synthetic dyes as food color additives induced damage to the liver tissue as evidenced by the significant increase in the enzyme activities of AST. ALT. LDH and γ-GT in serum of young administered rat groups. The observed disturbance in the liver function may be attributed to hepatocellular impairment leading subsequently to the leakage of the hepatic enzyme to the blood stream such a result which was in agreement with the finding reported by **[Abdel Rahim, et al. (1989)]**. Generally. the enzymes activities are considerably increased following the administration of various hepatotoxic compounds which leads to hepatocellular damage **[Grey et al., (1985)]**. In this study. the hepatic γ-GT activity showed a significant elevation. which considers a sensitive index for cell membrane damage, which may attributed. in part. to the effect of free radical generation induced by food colorants administration evidenced by elevation of lipid peroxidation and protein carbonyl contents.

The present data showed that food colorants administration in young rats either individually or in combination. leads to the kidney functional changes as detected by a significance elevation in the concentration of the serum urea. uric acid. creatinine and bilirubin. This result in agreement with **[Miller & Millstone (1987)]**, they found that

food colorants damaged the kidneys and adrenal tissues when fed to the laboratory rats.

Generally, relationship between oxidative stress and nephrotoxicity has been well demonstrated in many experimental animal models **[Maldonado et al. (2003)]**. Also, **[Pedraza-haverri et al. (2004)]** reported that, ROS produces cellular injury and necrosis via several mechanisms, including lipoperoxidation and protein modification.

Glutathione (GSH), a natural occurring antioxidant, protects the membrane polyunsaturated fatty acids from free radicals-mediated lipid peroxidation **[Kosower & Kosower (1979)]**. Therefore, the tissue level of GSH was considered a critical determinant for the threshold of tissue injury caused by several toxicant **[Meister & Taste (1976)]** depresses the glutathione leads to increased generation of hydroxyl radical and peroxidation of lipids and protein oxidation by altering mitochondrial respiration **[Ramsammy et al. (1985); Sha & Schacht (1999) and Maldonado et al. (2003)]**. It has been demonstrated that the depletion in the antioxidant tripeptide glutathione (GSH) may enhance the response of renal tissue to lipid peroxidation **[Othman et al., (2001)]**. In the present study, the results showed a decline in GSH contents, significant increase in TBARS (index of lipid peroxidation) and protein carbonyl (index of protein oxidation) contents in hepatic tissues in young rats administered food colorants daily for six weeks compared with the control rats, these results are consistent with the results obtained by **[Can et al. (2000) and Maldonado et al. (2003)]**. The decrease in GSH may be either due to the utilization of GSH in detoxification of free radical generated by food color additive administration or due to the inhibition of GSH-reductase activity.

It is known that the liver is an important organ for fighting against toxic injury of xenobiotics and endogenous toxins, in which ROS might be involved. Keeping the balance of ROS production and free radical scavenging plays an important role in maintaining the normal function of the liver. Also, ROS can damage proteins, lipids and DNA, playing a significant role in numerous diseases **[Adams & Odunze, (1991); Aebi (1984) and Ames et al., (1993)]**.

All the food additive colors inhibit mitochondrial respiration. This inhibition varied largely, from 100% to 16%, **[Reyes et al., (1996)]**. The over-stimulation leads to, initiating a cascade of events involving the activation of nitric oxide synthase (NOS), generation of free radicals and

mitochondrial damage **[Beal (1992)]**. ROS also mediates cytotoxicity of many environment chemicals. During the developmental period of synaptogenesis (brain growth spurt period). neurons are very sensitive to specific disturbances in their synaptic environment **[Olney (2002)]**. These investigations may explain the observed hyperactivity in the present study.

The results of the present study showed that hepatic hydroxy proline levels increased significantly in rats administered color additives. The most sever increase was observed in rats that administered the additive colors in combination. The observed data run in harmony with that obtained by the previous reports indicating that, liver damaged was characterized by increased deposition of collagen **[Friedman (1993)]**. Since. hydroxy proline is the major constituents of collagen (used as an index of collagen content) its increase parallels the increase of collagen deposition **[sugihara et al., (1999)]**.

The results in the present study. are also. in agreement with those of **[Anselmi et al., (2002); Nan et al., (2002) and Li et al., (2003)]**. In this regard. it was suggested that lipid peroxidation plays a role in the regulation of collagen gene expression. where there is a significance correlation between the hepatic thiobarbituric acid–reactive substances and hepatic collagenase.This later enzyme is used as a key indicator of intracellular collagen accumulation **[Brown et al., (1997)]**. So the present increase in the liver hydroxy proline content seemed to be related to the increased lipid peroxidation caused by the deleterious effect of color administration.

The results of the present study revealed that food colorants treatment induced a significant increase in serum iron; meanwhile the serum zinc concentration exhibited a significant reduction. According to the results obtained by **[Zhou et al. (2002)]**. as the severity of liver damage increases. the hepatic zinc decreases. The observed zinc depletion may be attributed to liver damage and decreased hepatic GSH contents. these results are in accordance with **[Loguercio et al. (2002)]** who reported that a direct correlation between zinc and GSH and pointed out that GSH levels were related to the degree of liver damage. Regarding serum iron. the significant elevation observed in the current study run in harmony with **[Halliwell & Gutteridge (1990)]** who reported that. iron has a major role in the initiation and propagation of lipid peroxidation. by either catalyzing the conversion of primary oxygen radicals to hydroxyl radicals or forming a perferryl ion. In addition, iron

can catalyze, directly lipid peroxidation and increase the oxidative reaction of polyunsaturated lipids, by removing hydrogen atoms from polyunsaturated fatty acids in the lipid bilayers of organelle membranes, it is now well established that oxidant stress itself can provide catalytic iron **[Halliwell & Gutteridge (1990)]**.

Electrophoresis of serum proteins, in the present study, showed that food color additives caused slight changes in most fractions of serum protein except fast green color which revealed a remarkable increase in albumin and globulin fractions especially gamma globulines as well as remarkable increase with combined colors after 6 weeks. These results are in agreement with the findings **[El-Sadany (1991)]**, in rats ingested the food colors indigo carmine and new coccin red color .Also the administration of tartrazine dye to the experimental animals caused a significant increase in their serum protein [**Lord (1992)**]. The observed elevations of gamma globulin fractions might be attributed to the increased immunoglobulin synthesis, the defense mechanism which aims to protect the body from the toxic effects of these synthetic food colors.

CONCLUSION

it must consequently be pointed out that most of the metabolic criteria were altered in rats subjected to the administration of the food color additives. These results have implications for the cellular effects of common chemical entities ingested individually (fast green color) or in combination. Other studies are recommended for revealing the hazards of food color additives and how we can avoid it.

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الملخص العربي

أجريت هذه الدراسة على ذكور الجرذان البيضاء (عمر ٢١ يوم فى بداية التجربه) لدراسة تأثير تناول الإضافات اللونية المستخدمة في صناعة الحلويات بمحافظة دميـــاط .تـــم اختيار ثلاث أنواع من هذه الملونات وهى:

New Coccin, Sunset yellow and **fast green dye**

لاختبار تأثيرها(منفردة أومجتمعه) على وظائف الكبد والكلـــى بالإضـــافة إلـــى تأثيرها على الإجهاد التاكسدى للدهون والبروتينات ومستوى الجلوتاثيون وتركيز عنـــصري الحديد والزنك .

وقد أظهرت الدراسة النتائج التالية:

- زيادة معنوية في وظائف الكبد و الكلى فى حيوانات التجارب المعاملـــه بالاصـــباغ اللونيه وخاصة المجموعة التى تعاطت الاصباغ الثلاثه مجمعة.
- زيادة معنوية في مستوى الإجهاد التاكسدى للـــدهون والبروتينـــات والهيدروكـــسى برولين في كبد الجرذان وخاصة المجموعة التى تعاطت الاصباغ الثلاثه مجمعة
- نقص معنوي في مستوى الجلوتاثيون الكبدي وتركيز عنصر الزنك فى مصل الدم
- زيادة معنوية في تركيز عنصر الحديد فى مصل الدم
- اضطراب في مستويات البروتين فى مصل الدم حيث تم فصلها عن طريق الفصل الكهربي .
- زيادة ملحوظة في النشاط الحركي للجرذان التي تناولت الملونات الخضراء Fast green والملونات المجمعة.

وومن كل ماسبق يتضح لنا من خلال نتائج البحث أن الإفراط في تناول الإضافات اللونية وخاصة المصنعة منها يؤثر تأثيرا كبيرا على صحة الفرد وخاصة الأطفـــال حيـــث يؤدى الى خلل فى وظائف الكبد والكلى والايض العام بالجسم لذا يجب منع هذه الملونـــات والاعتماد على الملونات الطبيعية .