RESEARCH ARTICLE

TOXICITY OF SUNSET YELLOW FCF AND TARTRAZINE DYES ON DNA AND CELL CYCLE OF LIVER AND KIDNEYS OF THE CHICK EMBRYO: THE ALLEVIATIVE EFFECTS OF CURCUMIN

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ABSTRACT

Recently, the use of synthetic food coloring agents were widely spread; and its potential toxic effects on human health were consequently increased. The present work aimed to assess the toxicity on DNA and cytotoxicity induced by in ovo administration of synthetic food coloring agents “sunset yellow FCF and tartrazine” with/without curcumin during the organogenesis phase of chick embryo at doses 1.575 mg/egg, 0.375 mg/egg, and 3.0 mg/kg eggs, respectively. The results showed that sunset yellow FCF and tartrazine increased in the level of DNA damage and the percentage of apoptosis. Also, there were a pronounced S phase arrest and a decrease in the number of cells in the G2/M of the cell cycle in the liver and kidneys of the chick embryo treated with sunset yellow FCF and tartrazine. On the other hand, co-administration of curcumin with the synthetic food coloring agents positively alleviated the changes in DNA and cell cycle distribution, as well as decreased the apoptosis.

INTRODUCTION

Food additives are any substance either natural or synthetic that added to the food in order to improve its palatability, shelf life, and texture [1,2]. They must be added in regulated quantities and should be within the acceptable daily intake (ADI), which provides large safety margin as it is the amount of a food additive that can be consumed daily over a lifetime without any adverse effect on health [1]. According to Amin and Al-Shehri [3], the food additive agents can be divided into preservative, flavoring, stabilizer and emulsifier, nutritional additives, and coloring agents. Food coloring is one of food additive agents that can be used in food, drugs, or cosmetics. There are two major categories of food colors; natural colors (exempt from certification) and synthetic colors (certified). It is used to offset colorless due to storage or processing of food, correct natural variation in food color, and enhance the appearance of food product to meet consumer demand [4].

Our previous studies have shown that some food colorants as sunset yellow (SY) FCF (E110) and tartrazine (Tz) (E102) caused many histopathological changes in liver and kidney of the chick embryo [5]. In addition, skeletal and morphological malformations were also noticed in the chick embryos-treated with SY and Tz [6]. Also,
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Gupta et al.\textsuperscript{(7)} found that erythrosine and Tz were teratogenic to zebrafish embryos. Moreover, some of food additives were proved to induce DNA damage in bacteria, fungi, insects, and mammalian cells \textit{in vivo} and \textit{in vitro}. They also cause chromosomal aberrations including mammalian cells\textsuperscript{(8)}. Moreover, some of them caused DNA damage \textit{in vitro} in human germ cells\textsuperscript{(9)}.

SY is a synthetic azo-dye, water-soluble, and orange-red colored powder that has been used in pharmaceuticals, cosmetics, and food industry\textsuperscript{(10-12)}. Joint FAO/WHO Expert Committee on Food Additives (JECFA) has established the maximum permitted level of SY for different types of food; the permissible limit of SY is 300 mg/kg food. Whereas, for water-based flavored drinks, the safety limit allowed is 100 mg/kg drink\textsuperscript{(13)}. The potential adverse effect of SY on human health is due to aromatic ring and azo-functional groups (\(-N = N-\)) in their structures, as it broken down by intestinal azo-reduction and most of dose is excreted in the feces and urine, and only little amount of it will be absorbed in gastrointestinal tract\textsuperscript{(10,13)}.

Tz is a synthetic organic azo dye, which made from petroleum products. It used in human food and drink, pharmaceutical products and cosmetics\textsuperscript{(14-16)}. The JECFA evaluated ADI to be 0.0-7.5 mg/kg/day\textsuperscript{(14,17)}. Tz can also be used in cotton candy, soups, jelly, ice cream, jam, sauces, hair products, and medical products like vitamins, antacids, and medical capsule\textsuperscript{(16,18)}. Tz is metabolized by the intestinal microflora into sulfanilic acid and aminopyrazolone-metabolites, which generate reactive oxygen species (ROS) leading to oxidative stress\textsuperscript{(15-17)}. Oxidative stress or generation of ROS has been closely linked to apoptosis\textsuperscript{(19)}.

Curcumin (Cur) (E100) or natural yellow 3 can be used as natural food coloring agent and the major yellow pigment extracted from turmeric. Turmeric derived from the rhizomes of \textit{Curcuma longa} family \textit{Zingiberaceae}\textsuperscript{(20)}. The major constituents of turmeric rhizomes are volatile oils and curcuminoids. The latter composed of curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] (77%), dimethoxy-curcumin (17%), and bis-dimethoxy-curcumin (3%)\textsuperscript{(21)}. It is a safe nutritional dietary supplement that has been widely used in traditional medicine and as a spice, coloring agent\textsuperscript{(22)}. The JECFA allocated an ADI of Cur is 0.0-3.0 mg/kg body weight/day\textsuperscript{(23,24)}. It is well documented fact that Cur has multiple activities such as ROS inhibitory, anti-inflammatory, anti-apoptosis, antibacterial, and anticancer\textsuperscript{(25-27)}.

Cur displays a pivotal role in diseases cure due to its effective scavenger to different forms of free radicals. It suppressed the generation of ROS and protected against DNA damage induced by benz(a)pyren or H\textsubscript{2}O\textsubscript{2}\textsuperscript{(28-30)}. Also, Cur has alleviative effect against the genotoxicity of betamethasone in the kidney and liver tissue of both rat mothers and their fetuses\textsuperscript{(31,32)}. The present study was performed to delineate the genotoxic and cytotoxic effect of two synthetic food coloring agents namely SY and Tz on the development of chick embryo, and to evaluate the efficiency of one natural color namely Cur in alleviating this toxicity.

**MATERIAL AND METHODS**

**Coloring agents’ administration**

SY FCF (E110) and Tz FD&C yellow n°5 (E102) pure powders were obtained from Kamina Co. (Cairo, Egypt). Both coloring agents (SY and Tz) were dissolved in sterile water and injected \textit{in ovo} (into the air sac) as single dose (0.2 mL/egg) at the 6\textsuperscript{th} day of incubation; the holes were carefully sealed with molten paraffin wax.

**Water extraction of Cur**

Dry rhizomes of the plant (\textit{Curcuma longa}) were bought from a herbal store at Shebeen El-Koom, Menoufia, Egypt and crushed into powder; 125g of the powder were macerated in 1000 mL of sterile water for 12 hours at room temperature and filtered through a 5 μm filter paper. The concentration of obtained extract was 24 mg/mL\textsuperscript{(33)}. Cur extract was applied at a dose of 3 mg/kg eggs\textsuperscript{(24)}. At the 6\textsuperscript{th} day of incubation, 0.2 mL...
of fluid was injected as single dose into the air sac; the holes were carefully sealed with molten paraffin wax.

**Egg incubation and grouping**

Principles of animal care and use were carefully followed during conducting the present study according to the guide for the care and use of laboratory animals approved by the Faculty of Science, Menoufia University, Egypt (Approval No. MNSE2208) and according to the National Institutes of Health guide for the care and use of laboratory animals (NIH publications No. 8023, revised 1978). Fresh fertilized chicken eggs (*Gallus domesticus*) were obtained from a local hatchery at Menouf, Menoufia governorate. Before incubation at 37°C in an artificial incubator, eggs were cleaned with distilled water and 70% ethanol (weighed 50 ± 5 g) and then labeled. To ensure the relevant humidity (65%), an open one liter container filled with distilled water was placed at the bottom of the incubator. The eggs were put horizontally and turned over, at least three times a day. Eggs were candled at the second day of incubation and the un-fertilized eggs were excluded. The remaining eggs were divided into seven groups (40 eggs/each group) and injected at the six day of incubation with a single dose, which is equivalent to 14 times ADI for synthetic colorants SY and Tz. A total of 280 fertilized eggs were used as follows:

- Group (A) was not subjected to any injection (control group).
- Group (B) was injected *in ovo* with 0.2 mL of sterile water (sham group).
- Group (C) was injected *in ovo* with 0.2 mL of Cur extract at a dose of 3 mg/kg eggs[^24].
- Group (D) was injected *in ovo* with 0.2 mL of SY at a dose of 1.575 mg/egg (ADI)[^34].
- Group (E) was injected *in ovo* with 0.2 mL of Tz at a dose of 0.375 mg/egg (ADI)[^14].
- Group (F) was injected *in ovo* with 0.2 mL of 1:1 mixture of Cur extract and SY at the same above doses.
- Group (G) was injected *in ovo* with 0.2 mL of 1:1 mixture of Cur extract and Tz at the same above doses.

**Embryo collection**

At the 20th day of incubation, the egg shells were broken with a scalpel and embryos were carefully freed. The living embryos were anaesthetized by light diethyl ether and dissected, and the target organs (liver and kidney) were immediately removed for subsequent analysis.

**Determination of DNA fragmentation**

Fresh liver and kidney samples (6 tissue samples/each group, each tissue sample weighing 10 mg) were used for nucleic acid extraction by DNA ladder detection kit according to the method originally introduced by Aljanabi and Martinez[^85] and modified by El-Garawani and Hassab El-Nabi[^36], in which the ethidium bromide direct staining of DNA samples were done. The collected DNA samples were frozen at −80°C until electrophoresed in 1.2% agarose gel to isolate DNA fragments.

**Cell cycle distribution analysis by flow cytometry**

Fresh tissue specimens (liver and kidney, 6 samples/each group) were transported to the laboratory of Center of Excellence in Cancer Research, Tanta University (Gharbia, Egypt) and prepared according to Tribukasit *et al.*[^37] for apoptotic assays by flow cytometry. Apoptosis was measured by using the sub-G1 peak staining with propidium iodide[^38], and data analysis was conducted using BD FACS DIVA™ software (BD Biosciences, San Jose, CA, USA), as shown in Figure 1.

**Data evaluation and statistical analysis**

All data sets were expressed as mean ± standard error of the mean. The data were statistically analyzed for normal distribution (independent samples t test) and homogeneity of variances (Levene’s test) using statistical package of social sciences (IBM SPSS) statistics software for windows,
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Version 22 (IBM corp., Armonk, NY, USA). Differences were considered insignificant whenever $P>0.05$. The significances of the obtained data were classified into three categories, i.e. $P<0.001$, $P<0.01$ and $P<0.05$ according to the obtained $P$ values.

**RESULTS**

**Curcumin alleviated the DNA fragmentation in hepatic and renal tissues of SY- and Tz-treated Chick embryo**

Figures “2a and 2b” revealed that the extracted total genomic DNA of the liver and renal cells of the control group was intact as seen in lane 1, with no damage of DNA. Also, administration of single dose of distilled water and Cur during organogenesis phase of chick embryo had no effect on the liver and renal DNA, as seen in Figure 2 (lanes 2 and 3, respectively). While, liver and renal cells of SY and Tz injected embryos showed marked DNA damage by the migration of DNA fragments, as seen in Figure 2 (lanes 4 and 5, respectively), compared with the control and sham groups (lanes 1 and 2, respectively). The liver and renal cells of SY+Cur and Tz+Cur treated groups showed some DNA fragmentation, but less than those of SY and Tz treated groups, as seen in Figure 2 (lanes 6 and 7, respectively).

**Figure 1:** Representative data for apoptosis and cell cycle analysis using flow cytometry: (a) Dot plots; (b) DNA histograms. PI: propidium iodide, Apop: apoptotic cells.

**Figure 2:** Photomicrographs of agarose gels showing evident variations of the DNA fragmentation in the liver (a) and kidney (b) tissue extracts of 20-days-old chick embryos treated with sunset yellow FCF and tartrazine with/without curcumin. Photo represented 6 samples/group. bp: base pair, M: marker DNA (100 bp DNA Ladder, New England Bio-labs, Ipswich, MA, USA), 1: control, 2: sham, 3: curcumin, 4: sunset yellow FCF, 5: tartrazine, 6: sunset yellow FCF+curcumin, 7: tartrazine+curcumin.
Curcumin alleviated the apoptosis and improved the cell cycle in hepatic and renal tissues of SY- and Tz-treated Chick embryo

The percentage of liver apoptotic cells of Cur group decreased significantly \((P<0.05)\) compared with the control and sham groups \((5.60±0.05, 6.00±0.09, 2.25±0.10, \) respectively, Figure 3). On the other hand, the percentage of liver apoptotic cells in groups treated with SY or Tz increased significantly \((P<0.001)\) up to 29.13±0.10 and 31.90±0.09, respectively, compared with the control group (Figure 3). While, co-administration of Cur with SY or Tz decreased significantly the percentage of liver apoptotic cells \((\text{up to } 2.13±0.08, 3.00±0.08, \) respectively) compared with SY or Tz treated groups that not received Cur \((P<0.001)\) and the control group \((P<0.05)\), as shown in Figure 3. In the control group, the majority of liver cells were in G0/G1 phase (Table 1). Meanwhile, in the liver cells of SY and Tz treated groups, a significant increase \((P<0.001)\) in cells of S phase was observed, which coincided with a significant decrease in cells of G0/G1 and G2/M phases \((P<0.001)\) compared with the control group (Table 1). By comparing the results of SY+Cur and Tz+Cur treated groups with those of SY or Tz alone treated groups, a statistically significant lower amount of S phase cells \((P<0.001)\) was observed, while the percentages of cells in G0/G1 and G2/M phases were significantly increased \((P<0.001, \) Table 1).

![Figure 3: Percentage of apoptotic liver cells of 20-days old chick embryos treated with sunset yellow FCF and tartrazine with/without curcumin. Data are represented as mean ± standard error of the mean \((n = 6)\). Cur: curcumin, SY: sunset yellow FCF, Tz: tartrazine. * and ***, \(P<0.05\) and \(P<0.001\), respectively, compared with the control group; †††: \(P<0.001\) compared with SY or Tz groups.](image)

**Table 1**: Phases of liver cell cycle of 20-days old chick embryos treated with sunset yellow FCF and tartrazine with/without curcumin.

<table>
<thead>
<tr>
<th>Groups</th>
<th>G1</th>
<th>S</th>
<th>G2M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>63.93±0.07</td>
<td>20.88±0.08</td>
<td>29.62±0.05</td>
</tr>
<tr>
<td>Sham</td>
<td>61.00±0.11</td>
<td>19.93±0.08</td>
<td>27.05±0.09</td>
</tr>
<tr>
<td>Cur</td>
<td>59.10±0.29</td>
<td>18.00±0.11</td>
<td>27.00±0.06</td>
</tr>
<tr>
<td>SY</td>
<td>6.80±0.09***</td>
<td>60.15±0.10***</td>
<td>15.00±0.06***</td>
</tr>
<tr>
<td>Tz</td>
<td>3.83±0.08***</td>
<td>57.02±0.06***</td>
<td>9.65±0.04***</td>
</tr>
<tr>
<td>SY+Cur</td>
<td>49.00±0.06**†††</td>
<td>22.25±0.13*†††</td>
<td>19.05±0.12**†††</td>
</tr>
<tr>
<td>Tz+Cur</td>
<td>41.90±0.09**†††</td>
<td>21.93±0.07**†††</td>
<td>17.63±0.08**†††</td>
</tr>
</tbody>
</table>

Data are represented as mean ± standard error of the mean \((n = 6)\). Cur: curcumin, SY: sunset yellow FCF, Tz: tartrazine. *, ** and ***: \(P<0.05\), \(P<0.01\), and \(P<0.001\), respectively, compared with the control group; †††: \(P<0.001\) compared with SY or Tz groups.
There were a significant increase (P<0.001) in the percentage of renal apoptotic cells in SY and Tz treated groups when compared with the control group (Figure 4). Co-administration of Cur with SY and Tz decreased significantly the percentage of renal apoptotic cells (P<0.001, compared with SY and Tz treated groups that not received Cur) up to the control values (Figure 4). In the control group, also the majority of renal cells were in G0/G1 phase (Table 2). However, groups treated with SY and Tz resulted in a higher percentage of arrested cells in the S phase (P<0.001), which coincides with a lower percentage of cells in G0/G1 and G2/M phases (P<0.001), compared with the control group (Table 2). Co-administration of SY or Tz in presence of Cur during the organogenesis phase of chick embryo displayed an evident ameliorative effect on cell cycle phases of the renal cells. In SY+Cur and Tz+Cur treated groups, a statistically significant decrease of S phase arrested cells (P<0.001) was observed with a significant increase in percentage of cells in G0/G1 and G2/M phases (P<0.001) compared with those of SY or Tz alone treated groups (Table 2).

**DISCUSSION**

The problem of the presence of food additives in an everyday diet and their possible hazard influence on human health is still discussed. So the present study is a trial to evaluate the genotoxic and
cytotoxic effects of two of the widely used synthetic food coloring agents. In the current study Cur did not increase DNA fragmentation in liver cells of chick embryos, while liver cells from embryos injected with SY or Tz showed a marked cellular apoptosis, as a result of DNA damage. Although apoptosis is involved in many normal biological processes, it can also be induced by chemicals. It is consider as a mechanism of programmed self-murder cell. It is described morphologically by condensation of nuclear chromatin, compacting of cytoplasmic organelles, cell shrinkage, and change at the cell surface[39]. Apoptotic cells in vivo are rapidly phagocytized, whereas in vitro rupture of the plasma membrane occurs only at late stage. Biochemically the most character of apoptotic cells were the fragmentation of DNA into oligonucleosomal fragments with lengths, which are multiples of 180-200bp[39]. The genotoxicity of azo dye compounds is dependent on their conversion to reactive metabolites, such as frequently produced N-acetylated forms. The activation is accomplished by acetyltransferases, which are widely distributed in animals[8,40]. Ali et al.[41] also found that oral administration of mixture of SY (as coloring agent) and sodium benzoate (as a food additives) in different concentrations to female rats for 12 weeks caused DNA damage in their hepatocytes, which confirmed by different types of comet assay. In addition, Hassan[8] reported that rats orally administrated two concentration of Tz (7.5 and 15 mg/kg body weight/day) for 7 weeks caused DNA damage in liver cells that increased by increasing the concentration. Moreover, Chen et al.[42] indicated that citric acid (as food additives) caused apoptotic DNA fragmentation in renal tissue of mice in a dose-dependent manner. Similar genotoxic findings were obtained by Abo-EL-Sooud et al.[47] who displayed that rats orally administrated with Tz at dose of 3 mg/kg body weight, daily for 60 days, caused moderate to high degree of DNA damage in renal cells. Cur was also effective in reducing the apoptotic DNA fragmentation in renal cells of chick embryos in the current study. Bayomy et al.[48] indicated that Cur inhibited the action of gentamicin-induced renal apoptosis, as result of DNA damage in renal tissue of rats. Cur also alleviated the degree of DNA fragmentation in rat fetal kidney after injection of betamethasone during organogenesis[31].

Cell cycle distribution analysis by flow cytometry was carried out to monitor the cell cycle progression and to study the effect of food coloring agents on both cell division and growth. Flow cytometry depends on measuring cellular DNA content for revealing the cell distribution with its phases and estimating the indecision of apoptotic cells with fraction DNA content[49]. The most clear feature of cell cycle are the
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synthesis and duplication of nuclear DNA before division, and the process of cellular division i.e. mitosis\[^{50}\]. The cell cycle consists of special phases G0 phase (quiescence), G1 phase, S phase (synthetic growth phases for many RNA and protein molecules that would be needed for DNA synthesis and cell growth before division), G2 phase (an interphase that was a time for repair of any DNA damage, which had occurred during the preceding cell cycle and for reorganization of the DNA structure before the DNA division between daughters cells), and M phase (mitosis, which subdivided into stages called nuclear morphology)\[^{51,52}\]. The traditional flow cytometry histogram analysis identified as G0/G1, S phase, and G2/M; couldn't able to distinguish between G0 and G1 phase, or G2 and M phase\[^{52}\]. The flow cytometry is able to measure the apoptosis as the cell visible as peak below G1 and usually Gaussian in shape and quantitated by histogram analysis. The present study revealed disturbance of the cell cycle and enhancement of cell death due to DNA fragmented of hepatocytes and renal cells of chick embryo among SY and Tz treated groups (cells arrested in S phase leading to a decrease in cellular division when compared with the control group). This is in agreement with Lye et al.\[^{53}\] who investigated the effect of three food dyes (SY, fast green FCF, and new coccin) on human liver cell line in vitro. They significantly affected the viability of cell and induced cell death. Also, Athinarayanan et al.\[^{54}\] showed that Silica E551 (as a food additives) induced a dose-dependent change in gene expression and cell cycle of human lung normal fibroblast. Meanwhile, Bhattacharyya et al.\[^{55}\] showed that Carrageenan (as a food additives) increased human IEC death in vitro and cell cycle arrest, and reduced cell proliferation. The present study demonstrated that Cur administration ameliorated the cell cycle distribution in hepatic and renal cells of SY and Tz treated chick embryo, as it decreased the percentage of cell death and the arresting in S phase of both hepatic and renal cells compared with the control group. It was reported that Cur can inhibit the chemotherapy-induced apoptosis in models of human breast cancer through its ability to scavenger ROS and inhibit the mitochondrial release of cytochrome\[^{56}\].

In conclusion, the synthetic colorants agents i.e. SY and Tz induced apoptosis and disturbance of cell cycle in liver and kidney when administered in ovo at doses 1.575 mg/egg and 0.375 mg/egg, respectively, during the organogenesis phase of the chick embryo. Furthermore, Cur administration alleviated significantly these genotoxic effects of SY and Tz in chick embryo. Therefore, it is essential to be aware of the hazardous effects of food additives, especially during pregnancy, and more attention should be focused towards using natural substitutes.

**CONFLICT OF INTEREST**
The authors declared that they have no potential conflict of interest.

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سُمية صبغيتي الأصفر غروبي والتآثرتين على الحمض النووي ودورة الخلية في كبد وكلي جنين الدجاج: التأثيرات المختلفة للكوركومين

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في الآونة الأخيرة، انتشر استخدام ملونات الطعام الاصطناعية، وبالتالي زادت تأثيراتها السامة المحتملة على صحة الإنسان. تم تنفيذ العمل الحالي لتقييم السمية على الدنا والدورة الخلية الناتجة عن حقن بيض الدجاج بصبغيتي الأصفر غروبي والتآثرتين مع أو بدون الكركم أثناء مرحلة تكوين الأعضاء وذلك بجرعات 1.575 ملم/بيضة و 0.375 ملم/بيضة و 3.0 ملم/كجم من البيض، على التوالي. وقد أظهرت النتائج أن حقن البيض بكل من ملونات الطعام الاصطناعية أدى إلى تكسر متعدد في الحمض النووي، وزيادة الموم المبرمج للخلايا، وكذلك توقف الدورة الخلية في المرحلة "S"، وانخفاض في عدد الخلايا التي عند الحد "G2/M" من دورة الخلية في كل من خلايا الكبد والكلي لجنين الفرخ. كما أدى الحقن المشترك للكركم مع ملونات الطعام الاصطناعية إلى التخفيف بشكل إيجابي من التغيرات في الحمض النووي وتوزيع الخلايا في الدورة الخلية، وإلى تقليل الموم المبرمج للخلايا.