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Cytotoxic effect of commonly used food dyes on human hepatoma cell line, HepG2.

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Abstract

The increasing and widespread use of synthetic food dyes raises health concerns and earlier reports suggest that certain food dyes might be harmful for human health. In this study, we have investigated the effect of three commonly used food dyes on human liver cell line, HepG2. Our findings suggest that these experimental food dyes significantly affect cell viability and this effect can be worsen in hyperglycemic condition. Accumulation of cellular fat was significantly higher in presence of these dyes. Expression pattern of the gene involved in regulating apoptosis suggests that that the observed cell death could be attributed to the activation of apoptotic pathway. These findings suggest that these experimental dyes might exert synergistic toxicity in hyperglycemia that need to be confirmed using suitable *in vivo* models.

Introduction

Over the past few decades, food additives and preservatives added a new threat to population health. These are often associated with highly prevalent non-communicable diseases. Obesity, cardiovascular diseases, and cancer are the leading cause of mortality and main disease factors that cause non-communicable disease related death all over the world. In case of obesity and obesity-related cardiovascular diseases, consumption of carbohydrate, mainly sucrose, is undoubtedly contributes to the development of these pathological conditions. Several reports suggested that carbohydrate-mediated health effect can be further deteriorate in presence of other dietary ingredients such as lipids. While several food additives and food preservatives are known to exert toxicity in experimental models, their combined effect along with high level of glucose has not been established. Synthetic food dyes are widely used food additives in the food and beverage industry as well as in non-food applications such as in pharmaceutics, cosmetics and in the medical industries. Food industries use the colouring mainly to increase aesthetic appeal and palatability to the consumers as consumers usually incorporate perception based on sight when judging a food product, where they tend to associate certain colours with specific foods (Burrows, 2009). Food processing that is conducted in large scales also necessitated the need for food dyes to be added to ensure uniformity of the appearance of these foods (McAvoy, 2014).Hence, it should come as no surprise that food and beveragemanufacturers include these dyes as a food additive in their products in order to appeal to the mass market. This widespread usage of food dyes across industries has also led to the development of a range of colours and in several forms, including powders, liquids and ready-to-use pastes and gels for use in different applications.

Although the usage of dyes is well regulated, it is difficult to ascertain the total amount of dye a person consumes, especially since there could be additive and synergistic effects of consuming more than one dye in one sitting, which is becoming increasingly common due to the wide availability of processed foods. Several studies have also attempted to investigatethe possible toxic effectsof such synthetic dyes(Ashida *et al*., 2000; Tsuda *et al*., 2001; Sasaki *et al*., 2002; El-Wahab and Moram, 2013)which could potentially lead to organ damage, birth defects, various types of cancers and immune reactive disorders (Vojdani and Vojdani, 2015) as well as behavioural issues in children(McCann *et al*., 2007; Kobylewski and Jacobson, 2010).A recent report suggests that Sunset Yellow FCF and Brilliant Blue FCF are able to alter mitotic index and induces

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micronucleus in lymphocyte which ultimately lead to genotoxicity and cytotoxicity (Kus and Eroglu, 2015). Artificial food coloring has also been implicated in developing allergic and other immune reactive disorders. The ingestion of food delivers the greatest foreign antigenic load that challenges the immune system. These micro-molecules are easily absorbed by the cells, are often bound to the food particles, and are able to bind to cellularproteins that might have significant impact on our immune system. Hence, their consumption can activate the inflammatory cascade and autoimmunity (Vojdani and Vojdani, 2015).

We have attempted to investigate the toxicity of three commonly used food dyes, Fast Green FCF, Sunset Yellow FCF and New Coccine, which are used in a myriad of food and drinks.Fast Green FCF, also known as C.I. 42053, FD and C Green #3 and Food Green 3, is a triarylmethane dye while Sunset Yellow FCF is an azo dye derived from petroleum and is also known by other names, including E110, FD and C Yellow #6, C.I. Food Yellow 3 and Orange No. 2. New Coccine, also known as cochineal red, E124, ponceau 4R and C.I. Food Red 1625, is an azo dye used to produce a red color.

The aim of our research is to study the cytotoxic effect of commonly used food dyes on human hepatoma cell line. We have used human liver cell line, HepG2, as an in vitro model since liver is the major organ responsible for filtration and detoxification of toxins that are ingested. Several experimental strategies have been employed to investigate the properties of these dyes in affecting cell survivability, cellular lipidaccumulation, and expression of certain key genes that affect these processes.

Materials and Methods

Food dyes

Three different food dyes, Fast Green FCF (CAS No. 2353-45-9), Sunset Yellow FCF(CAS No. 2783-94-0) and New Coccine (CAS No. 2611-82-7) were obtained from Sigma-Aldrich (Missouri, USA) to be used in this study.These dyes are of biochemical grade and free from additives such as stabilisers, carriers and emulsifiers found in commercial dyes. The dyes were dissolved in phosphate-buffered saline (PBS) with a pH of 7.4 (Gibco, New York, USA).

Cell culture and treatments

Human liver carcinoma cells, HepG2(ATCC HB-8065) are used in this study as an in vitro model. The cells were culturedin Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, sodium pyruvate, and non-essential amino acids (all from Gibco, New York, USA)in a water jacket incubator at 37°C and in the presence of 5% carbon dioxide (CO_2). The cells were exposed to the three food dyes at concentrations between 75 µg/ml to 300 µg/ml in each of the experiments. The control consisted of DMEM and phosphate-buffered saline (PBS) with a pH of 7.4 (Gibco, New York, USA).

Cell viability assay (MTS)

Cell viability was measured by MTT-based MTS reagents,CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay(Promega,Wisconsin, USA) according to the manufacturer's protocol. 14 to 16 hours before the treatment, 5000 cells per well were platted in 96-well plates containing 100 µl of culture medium and treated with food dyes as indicated in the figure legends. To analyze cell viability, 20 µl of the MTS solution mix was added to each well and the plates were then incubated at 37°C in a humidified (5% CO_2) incubator until it developed desired color. This was monitored every 15 minutes. Absorbance of the reaction mix was measured at OD490nm using Varioskan Flash Multimode Reader (Thermo Scientific, Massachusetts, USA).

Oil red O staining

To detect intracellular lipid accumulation, the cells were cultured in 24-well plates according to the experimental conditionsindicated in the figure legends. After treatment, the cells were washed with 1X PBS and then fixed with 10% formalin for one hour at room temperature. The cells were then washed twice with distilled water followed by 60% isopropanol for 5 minutes at room temperature and let it dry at room temperature. Once dried, the cells were stained with 0.21% Oil Red O solution (Sigma-Aldrich, Missouri, USA) for 10 minutes followed by washing with distilled water for 4 times and let it dry at room temperature. 100% isopropanol was added to each well and incubated for 10 minutes under gentle shaking to elute lipid bound dye. The intensity of oil-red-o measured using a microplate reader at OD500 nm.

Extraction of RNA from cultured cells

The cultured cells were washed with sterile PBS twice before lysed in 1.5 ml of QIAzol Lysis Reagent (Qiagen, Germany) and incubated at room temperature for 5 minutes. The lysate was transferred intoa 2 ml microfuge tube. 300 µl of chloroform was added into each tube and centrifuged at 12,000 x g for 15 minutes at 4°C. The supernatant was

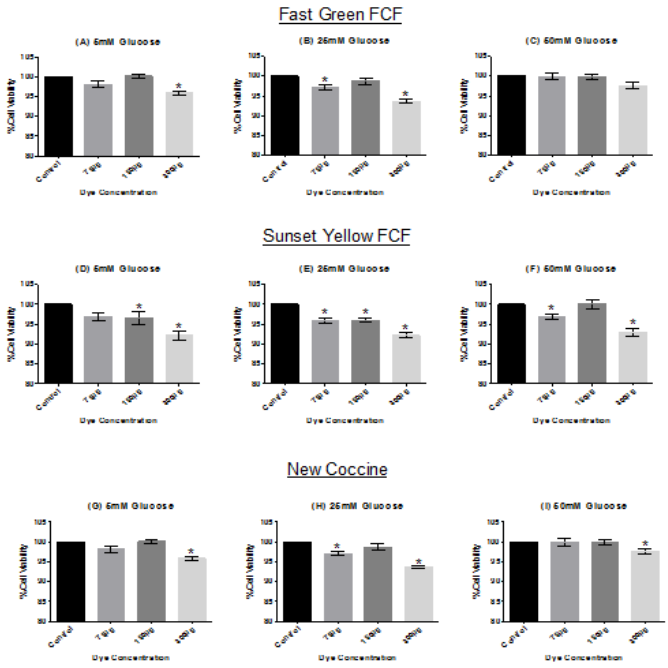

Figure 1. Effect of 48-hours treatment with 75 µg – 300 µg (A-C) Fast Green FCF, (D-F) Sunset Yellow FCF and (G-I) New Coccine dyes on HepG2 cells cultured in DMEM with glucose concentrations of 5 mM, 25 mM and 50 mM. Control consisted of DMEM at the respective glucose concentration. MTS assay was carried out and absorbance read at 490nm. Viable HepG2 cells are expressed as a percentage of the untreated control group. All data are expressed as mean±SEM (n=8).*, significantly different from control, P<0.05.

transferred to a new microfuge tube and mixed with 0.75 ml isopropanol. The RNA was precipitated at room temperature for 10 minutes followed by centrifugation at 12,000 x g for 10 minutes at 4°C. The supernatant was discarded and RNA pellet was washed once with 75% ethanol.

Real time PCR

1ststrand cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Cat. No. 205311, Qiagen, Germany) following manufacturer's protocol. Briefly, 2.5 µg of total RNA was mixed with 2 µl of gDNA buffer and top up to 14µl with RNase-free water. After 2 minutes' incubation at 42°C, the sample mix was chilled on The reverse-transcription master mix was added and the reaction volume was brought up to 20 µl. This reaction mix was incubated at 42°C for 30 minutes and finally the reverse transcriptase was inactivated by heating the samples at 95°C for 3 minutes. The prepared cDNA was used as a template for real time PCR using QuantiFast SYBR® Green RT-PCR Kit (Cat. No. 204154, Qiagen) which was performed according to the manufacturer's protocol. Briefly, 1 µl of 1st strand cDNA (prepared previously), 0.5 µl of each set of primers and 10 µl of QuantiFast SYBR® Green RT-PCR Master Mix were mixed and topped up to a total reaction volume of 20 µl with DNase free water. The cDNA was amplified and quantified using Eco Real-Time PCR System (Illumina, California, USA). Primer sequences are as follows:

Bax: For 5'-CAGATGTGGTCTATAATGC -3', Rev 5'- CTAATCAAGTCAAGGTCAC -3';
Scd1: For 5'- TGGGTGGCTGCTTGTG -3', Rev 5'- GCGTGGGCAGGATGAAG -3

Statistical analysis

We have used SPSS statistics software for statistical analyses where the significance of the treatment groups as compared to the control group was determined using one-way ANOVA and post-hoc Dunnett's test. The mean was considered significantly different if the p-value was less than or equal to 0.05.

Results

In our initial experiment, we have measured cell viability upon treating them with three dyes (Fast Green FCF, Sunset Yellow FCF and New Coccine) at concentrations of 75 µg/ml, 150 µg/ml and 300 µg/ml for 48 hours (Figure 1). Cells were cultured in medium containing three different concentrations of glucose; 5 mM, 25 mM, and 50 mM to mimic

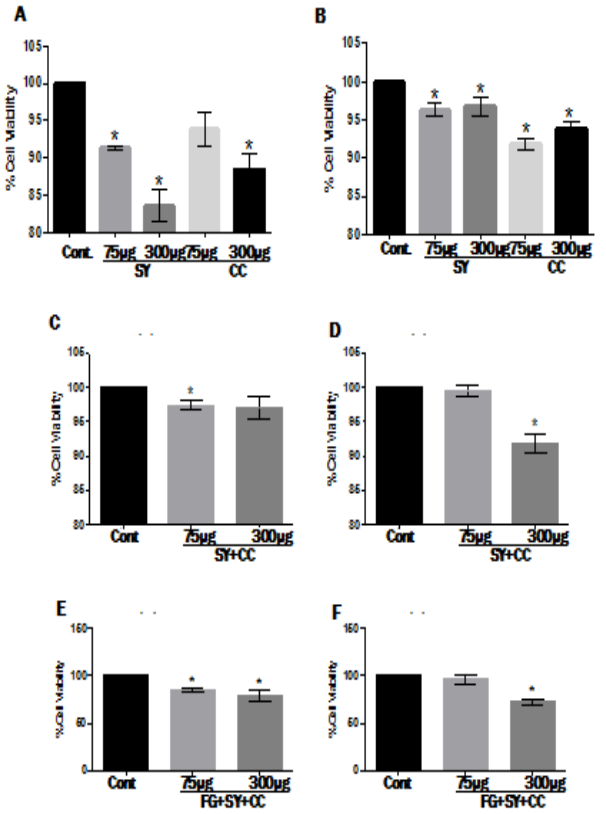

Figure 2. Effect of 72-hour treatment with Sunset Yellow FCF (SY) and New Coccine (CC) dyes at concentrations of 75 µg/ml and 300 µg/ml in 5 mM (A) and 40mM glucose containing medium (B). Cell viability was measured as percentage relative to the control. Middle panel shows the effect of combined treatment of Sunset Yellow FCF (SY) and New Coccine (CC) in 5 mM glucose (C) and 40mM glucose containing medium (D). Bottom panel shows the results obtained from treating the cells with three dyes: Fast Green FCF (FG), Sunset Yellow FCF (SY) and New Coccine (CC) in 5 mM glucose (E) and 40 mM glucose (F). All data are expressed as mean ±SEM (n=8). * P<0.05.

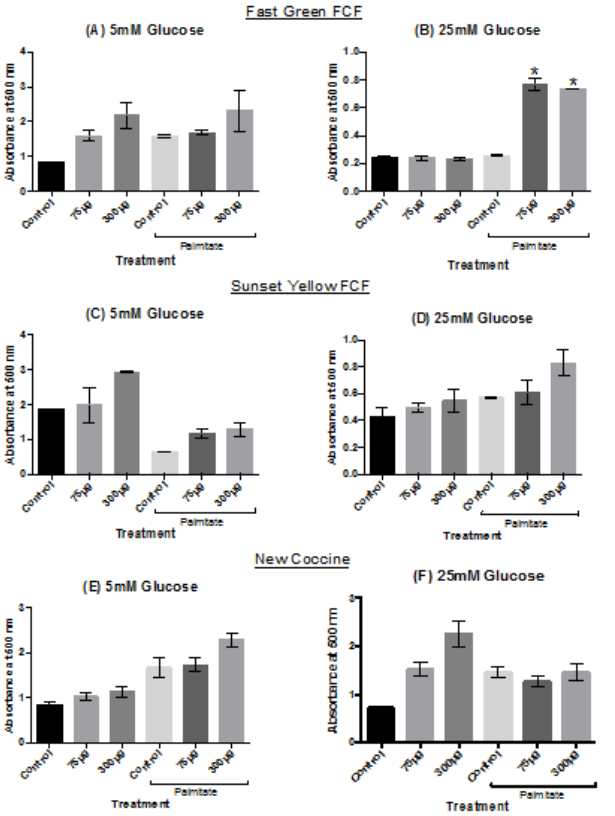

Figure 3. Effect of Fast Green FCF (A), Sunset Yellow FCF (B), and New Coccine (C) on intracellular lipid accumulation in presence of low or high glucose. After 72hours of treatment, intracellular lipid accumulation was measured. All data are expressed as mean±SEM (n=3). * P<0.05

hypoglycemic, normal, and hyperglycemic state respectively. Our MTS cell viability assay suggests reduced cell viability upon treating this human liver cell line. The survivability of the cells treated with Fast Green at a concentration of 300 µg/ml was significantly lower both in 5 mM and 25 mM glucose containing medium (Figure 1A and1B) while 75 µg/ml affected cell growth significantly only in presence of 25 mM glucose (Figure 1B). Sunset Yellow hinder cell growth in all the experimental units except 75 µg/ml dye in 5 mM glucose and 150 µg/ml in 50 mM glucose containing DMEM (Figure 1D, 1E and 1F). New Coccine exhibited similar trend with Fast green where cell survivability was significantly lower in presence of 300 µg/ml of this dye in all glucose concentrations and in 75 µg/ml of New Coccine in 25mM of glucosecontaining medium (Figure 1G, 1E and1F). As stated earlier, the cell viability was measured after 48 hours of treatment in this initial part of experiment.

In order to observe the difference after longer time point, we have investigated cell viability after 72 hours of incubating the culture in presence of these experimental food dyes. The MTS assay was carried out with only SY and CC at concentrations of 75 µg and 300 µg in a low glucose concentration medium (hypoglycemic) of 5 mM and a high glucose medium (hyperglycemic) of 40 mM, as shown in Figure 2.In low glucose concentration, the reduced cell viability is correlated with the increase of the dye, percentages of cell survivability are 91% and 83% for 75 µg and 300 µg of SY, respectively whereas survival rates for CC were 93% and 87% for 75 µg and 300 µg of the dye used (Figure 2A). In hyperglycemic conditions(40 mM glucose),however, the cell viability for both dye concentrations showed a similar trend, 75 µg and 300 µg of SY or CC did not show any significant difference but the cell viability was consistently lower in CC treated group compared to the control (Figure 2B). Subsequently we have investigated if a combined mixture of the dyes studied would produce any different effects on cell viability;

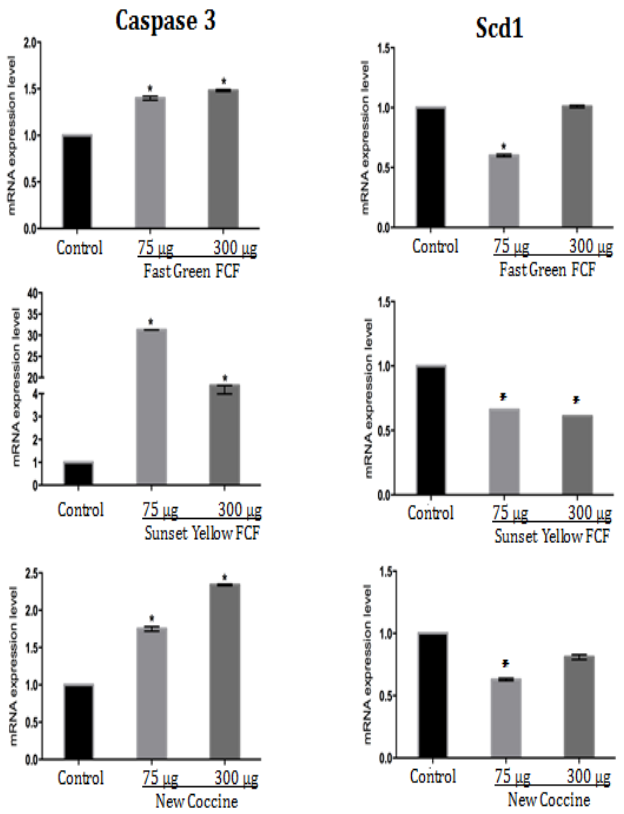

Figure 4.Effect of the dyes on the expression of Caspase3 and Scd1 mRNA as measured by real- time PCR. All data are expressed as mean±SD (n=3). *P<0.05.

we treated the cells with combined two dyes (SY and CC) and three dyes (FG, SY and CC) for 72 hours. Consistent with previous experiments, two different glucose concentrations were used, i.e.,hypoglycemic (5 mM) and hyperglycemic (40 mM) and the cell viability was measured using MTS assay (Figure 2C-2F). In all the experimental groups, cell viability was consistently lower in the dye-treated groups compared to the control with a trend of decreased cell viability with increased dye concentration (from 75 µg to 300 µg).

To assess the effect of these dyes in inducing lipogenesis or accumulating fat in vitro, we have cultured the cells in presence of saturated fatty acid (palmitic acid) and treated with these three experimental dyes. Cellular lipid level was measured using Oil Red O staining that stained intracellular lipids (Figure 4). This experiment suggests that there is a general trend of an increase of intracellular lipid level as detected by eluting the stained Oil Red O dye and measuring absorbance at OD 500nm. Lipid accumulation increases with the increase in food dye concentrations in both 5 mM and 25 mM glucose containing media.

Programmed cell death or apoptosis is one of the well-established mechanisms of stress-induced cell death. As we have observed that cell survival was significantly reduced in presence of these experimental dyes, we investigated the expression pattern of caspase 3, a key gene, involved in apoptosis to understand the mechanism of cell death. As shown in Figure4, expression of pro-apoptotic Caspase 3 is elevated in presence of the dyes (Figure4, left panel). These observations suggest that the experimental dyes might induce genotoxic stress that triggers the apoptotic pathways and induce cell death. Stearoyl-CoA desaturase-1 (SCD-1) plays a pivotal role in accumulating triglyceride in hepatocytes. To confirm our Oil-Red-O staining experiment, we have measured expression of Scd1 in these cells and we have observed that Scd1 expression is downregulated at low concentration of these dyes, but increased in a dose dependent manner (Figure4, right panel).

Discussion

Food dyes and preservatives are integral part of modern foods and beverages. Increasing incidence of several non-communicable diseases globally warrants a precise study on the food ingredients as most of these diseases are either diet-related or at least influenced by diet. A number of reports have suggested that apart from preservative, the food dyes might have harmful effect on human health too. In this study, we have investigated toxic properties of three commonly used food dyes both in food industries as well as in pharmaceuticals. Since liver functions as the major organ for detoxification of substances ingested by the body, including drugs and potential toxins, we have used human liver cell line (HepG2) as an in vitro model. Hepatocytes are the most abundant cells (around 70-80%) in the liver and abnormalities in hepatocytes are mainly responsible for liver disorders (Wang, 2014).

Although the three food dyes used in this study are currently approved for use at their respective ADI levels by the FDA (FDA, 2015), the potential cytotoxicity of these and other food dyes has been consistently reported over the years. A recent finding suggest that Sunset Yellow FCF may contribute to cholestatic injury and when combined with other factors could lead to the development of primary biliary cirrhosis, a cholestatic liver disease(Axon *et al*., 2012).Shimada *et al.* (2010) showed that colonic DNA in mouse was damaged when New Coccine was administered. However, insufficient studies with small sample sizes and study limitations in most cases have made for inconclusive evidence. In this study, cells were cultured in hypoglycemic and hyperglycemic conditions in vitro to mimic the human physiological conditions of hypoglycemia and

hyperglycemia respectively. These cells were treated with single food dyes of different concentrations ranging from 75 µg to 300 µg and our findings from the MTS assays carried out (Figure 1 and 2) suggest that the three food dyes studied exhibit cytotoxicity as cell viability was reduced.

This study also investigated if there were any effects of the food dyes on the process of lipogenesis in hepatocytes, as the incidence of hepatic steatosis has been steadily on the rise in recent decades. Lipid peroxidation, mitochondrial dysfunction and oxidative stress are all known mechanisms of hepatic steatosis, which could lead to liver injury (Browning and Horton, 2004). Oil Red O staining, which selectively stains cytosolic lipids, was carried out on the treated cells (Figure3). This observation suggests that the addition of food dyes to the cells induced lipid accumulation, even without the presence of free fatty acids. The levels of accumulation of fat were different in hypoglycemic or hyperglycemic conditions and the types of food dyes used. However, the overall trend of increasing lipid accumulation with increasing dye concentration is apparent. These findings thereforeraised the possibility that the food dyes are capable of inducing lipogenicity which can lead to lipid-mediated liver disorders such as hepatic steatosis or its advanced forms(Browning and Horton, 2004). In order to confirm the findings of the MTS assay on cell viability, a subsequent experiment was carried out using RT-PCR to determine the molecular mechanism of cell death. The relative mRNA expression levels of several genes involved in the regulation of apoptosis were tested as shown in Figure4. Apoptosis, or programmed cell death, involves two major apoptotic pathways; the intrinsic (or mitochondrial) pathway, and the extrinsic (or death receptor) pathway (Elmore, 2007).In both pathways, caspase (Cas-3) is the executioner caspase that induce activation of downstream inducers of cell death such as degradation of cytoskeletal proteins, nuclear membrane, degradation of DNA, etc. Cas-3 levels are significantly upregulated in all three food dyes (Figure4) it can be speculated that genotoxic stress due to the presence of the food dyes induce cell death in our experiment cell line. Studies have demonstrated that when Scd1 is suppressed, the rate of cell proliferation is reduced, accompanied by a decrease in survival rates(Igal, 2010). Scd1 is also involved in regulation of lipogenesis, where an overexpression of Scd1 prevents the accumulation of saturated fatty acids from occurring. This is done through several mechanisms, including modulating the signaling networks and enzymes involved in lipid metabolism, controlling the rate of fatty acid biosynthesis and controlling the provision of monounsaturated fatty acids for the formation of lipid macromolecules (Igal, 2010). Thus, the reduction in Scd1 seen in all three dyes (Figure4) is in agreement with the suggested cell death and accumulation of lipids as seen in the findings of this study.

Toxic effects of food colorings are known for long time and it is well established that a number of these dyes exert cytotoxic effect that might lead to gene mutation and other xenotoxic stresses. We have mainly focused on the dyes that commonly used in food products elsewhere. In this study, we have observed that these experimental dyes might affect lipid metabolism or its homeostasis apart from their known toxic effect. We have used human liver cell line since liver is the primary organ that deals with the detoxification of dietary ingredients. Future research on an in vivo model might address the effect of these dyes on developing fatty liver diseases and its advanced forms. Investigating the potential of these dyes in inducing lipogenesis and lipid metabolism will help to assess their acceptability to use in food industries. An *in vivo* model will be also useful address the molecular mechanisms that drive these effects.

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Declaration of conflict of interest:

The authors declare that there is no conflict of interest.

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