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Adverse Effect of Azo Food Colourants: A Toxicological Aspect

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Abstract

Food colourants are important food additives which not only enhance the appearance of food, but also appetite and hold a very significant position in the food industry. These can be obtained from natural and synthetic sources, but synthetic sources are more popular, efficient and potential in its functional terms. Although food colourants don't have any nutritional value, but their quantity and quality present in food materials imparts serious health risks. Earlier, studies reported that azo food colourants are injudiciously used in the food products and also developed suitable analytical methods for their detection in food materials. Further, evidences from various literatures suggested that food colourants which contains azo dyes are the causal factors for neurotoxicity, carcinogenicity, genotoxicity, hypersensitivity and therefore not safe for the human consumption. The present review article has been aimed to evaluate the toxicological profile of azo food colourants and associated adverse health impact. The findings of the study will add insightful facts to the literature and help the regulatory agencies to revisit and improve their toxicity testing procedures and exercise greater caution regarding continued approval of these dyes, and in the future approve only well-tested and, safe dyes.

1. Introduction

The Food Colourants (FCs) are any dye, pigments, or chemical substances that impart colour or formed colours added to food, drink, pharmaceuticals, etc.[1,2]. Food products are artificially coloured to make attractive mixtures of basic ingredients to lure consumers because it not only attract but also enhance appetite. FCs are classified into natural and synthetic colourants where natural colourants could be obtained from animals, plants,

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and microorganisms. They have limited application in food industries because of many disadvantages including high cost, poor colouring ability, and ease of discolouration. In contrast, synthetic colourants are brightly coloured and stable, so they are extensively employed over natural FCs in food industries. Different countries and regions have their strict legislations for types, application scopes, and limit standards of the synthetic FCs that are approved for human consumption. Because of the few varieties and the relatively high price of the approved natural and synthetic FCs, industrial food colours with the features of strong colouring ability and low price have been illegally added in food products by some vile food manufacturers[3]. Synthetic FCs often have high toxicity and adverse effects, including teratogenic, carcinogenic, and mutagenic effects. Thus, ingestion of the foodstuffs containing the non-permitted FCs poses great risk to consumer's health[4]. The large number of food safety incidents caused by the illegal addition of non-permitted FCs in the past decades have received attention as one of the major issues of food safety all over the world.

Approval of FCs as additives depends on the available scientific knowledge on technical and chemical specifications of the colourant, description of the manufacturing process, analytical methods used for the determination, chemical reactivity with food, dose range, toxicokinetic information, toxicological data, such as, carcinogenicity, genotoxicity, developmental, and reproductive toxicity and hypersensitivity to the substance (Figure 1). Experts on reviewing toxicity of FCs suggested that, numerous azo FCs significantly decreases in erythrocyte count, hemoglobin concentration, and hematocrit value**,** whereas some of the azo FCs can significantly increase thyroid-stimulating hormone concentration, which indicate a primary thyroid abnormality[5]. For this reason food safety authorities summaries all possible data which concern from health point of view, and suggests either it permeable or not. The toxicological data on FCs must be confirmed in 6 animal species, and 3 out of them must be mammalian which are close to the human body physiology. Preclinical data from animal studies actually confirm the No- Observed-Adverse-Effect Level (NOAEL). NOAEL quantity then

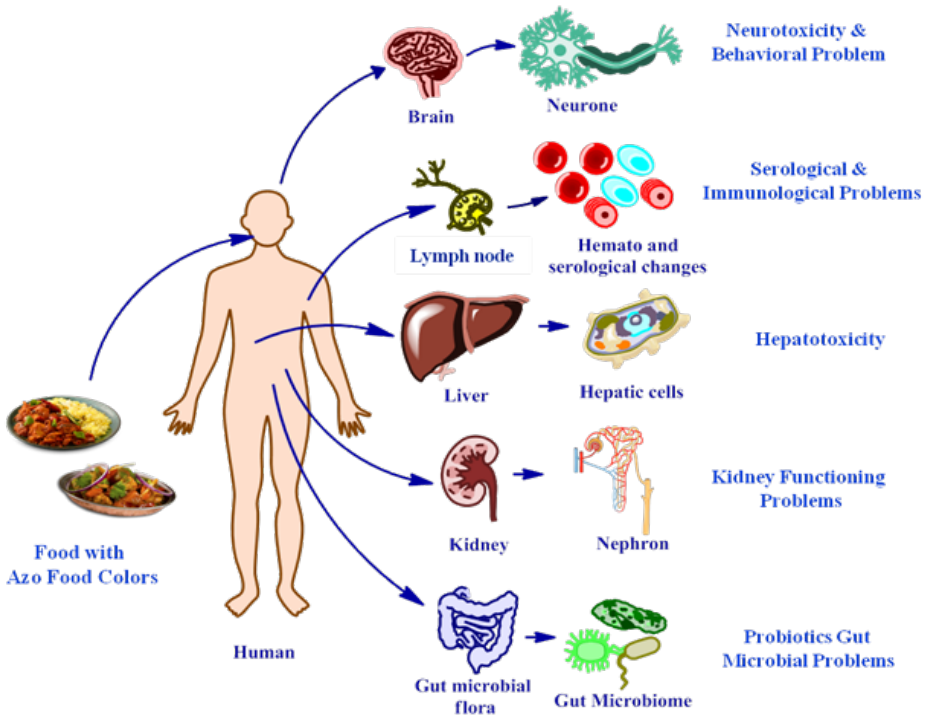


Figure 1. Effects of azo FCs on different system of body and associated health effects.

divides by safety factor, usually is 100 to determine the Acceptable Daily Intake (ADI)[6]. Such precautions and care, even some time cannot completely eliminate the risk of possible adverse reactions to a particular substance, especially with regard to vulnerable populations or hypersensitive individuals.

In spite of strait guideline on ADI for permitted food colourants and strict prohibition of banned azo food colourants, a concern raised time to time regarding their human health effects in the studies where higher ADI value for permitted food colourants and illegal use of banned food colourants are reported[7–10]. Besides human health concern, they are relatively persistent pollutants as their higher stability, hindering the removal or reduction of toxicity of dye containing industrial wastewaters by conventional treatments. About 15–50 % of the total amounts of dyes used during manufacturing in industries are released into the aquatic environment, and azo dyes are also toxic to freshwater organisms[11]. The number of food colourants banned for its toxic effects in recent years as at the time of their approval all the data were not available and they were effectively rum into the market. The toxicity due to FCs reported here are mostly synthetic in nature, permeable or non-permeable, and either higher or lower than in their ADI range. The present review article has been aimed to evaluate the toxicological profile of azo FCs in the general context like allergic reaction, hematological alterations, effect on gut microbiome, liver, kidney, spleen, brain and DNA damage and other adverse health impact.

2. Allergic Reactions

Allergy is a kind of adverse reaction mediated by any immunogenic moiety in which immune mechanism either specific immunoglobulin E (sIgE) (IgE-mediated), or cell mediated (non-IgE-mediated) or both IgE and cell-mediated mechanisms are involved. Several literatures support that consumption of FCs can induce food intolerance (non-immune mediated) and allergic reactions (immune mediated, ranging from urticaria and/or angioedema, and asthma to anaphylaxis)[12]. Sometime intolerance, which is not directly involved immunogenic mechanism but mimic reactions typical of an immune response include metabolic, pharmacological, and toxic or undefined mechanisms can be confused with allergy. Here FCs can act as immunogens that elicit an immune response only when covalently bound to a large carrier, typically a protein antigen[13]. Possible mechanism associated with the FCs allergic reaction involves an immune mechanism, and non-allergic reactions[14]. Synthetic FCs do not contain protein content so true allergies are fortunately very rare, that's why the reaction to the FCs classified as intolerances or sensitivities. Symptoms exacerbated by the synthetic FCs usually involve a non-IgE-mediated mechanism (i.e., pseudo-allergic reaction), and are typically less severe rather than true allergy[15]. Mostly synthetic FCs are act as haptens which bind with protein food content to induce IgE-mediated response. So FCs indirectly activates specific components of the immune system, leading to histamine release, and increased leukotriene production. T cells activate releasing of granules and cytokines by mast cells when get stimuli by the hapten protein complex which results in trigger typical symptoms of allergy, and an increase in IgE levels[16].

Earlier studies showed an increase in plasma histamine level, decrease in cyclo-oxygenase metabolism and consequent production of prostaglandin which suggests the probable mechanism of tartrazine[17]. The study suggests that very small dose of azo FCs able to absorb from the skin and can trigger the immune response[18]. Clinical study on 100 patients with chronic idiopathic urticaria (CIU) showed only 2 patients had positive response on single blind challenge to tartrazine and sunset yellow, but on double blind, placebo-controlled food challenges neither patient reacted, which indicate that the prevalence of tartrazine and sunset yellow sensitivity in adult patients with CIU was estimated to be less than 1%[19]. Thus the prevalence of allergic reaction due to FCs is unknown, but it can consider being low which likely to elicit an allergic reaction in patients with atopic disease than healthy. Studies have documented the association of FCs with adverse skin reactions, such as urticaria and angioedema, and aggravation of pre-existing atopic dermatitis but evidence of a causal relationship is not available. It has also been found that FCs can be correlated with an increase in the incidence of allergies and asthma[20]. A recent study conducted in Singapore in *in-vitro* effects of five azo FCs, tartrazine, sunset yellow, carmoisine, allura red, and ponceau 4R effects on the *in vitro* synthesis of leukotriene B4 and F2-isoprostanes on freshly isolated blood neutrophils. All five FCs showed

significant increase in the formation of leukotriene B4, and F2-isoprostanes eliciting pro-inflammatory responses *in vitro,* suggests potential health risk detrimental to the local population[21]. Besides azo synthetic FCs, non-azo dyes like erythrosine, brilliant blue, indigotin, and patent blue also exacerbate the symptoms of allergic reaction[22].

3. Bio-activation of Azo FCs through Gut Microbiome

The mutual interaction between human and microorganism evolved from long time due to symbiotic relation, as human beings to influence the host physiology. More than 10^{22} bacterial cells of different species have been found in the human gastrointestinal tract, and these populations are 10 times higher than human body cells. Different species of microorganism from the mouth opening to end of the digestive tract has populated, but the largest diversity of the human associated microbes is found in the intestinal tract with about 10^{13} per gram in stool[23]. It has been observed that, most of the intestinal bacteria are members of the Gram positive *Firmicutes* and gram negative *Bacteroidetes* phyla. The other relatively few (less than 10%) intestinal bacteria belong to *Proteobacteria, Actinobacteria, Fusobacteria,*

Table 1. Biotransformation of azo food colourants into toxic metabolites

Azo Food Colourants	Metabolites	Group[(a)]	Reference
Orange G, 1-phenylazo-2-naphthol, 1-[(2,4-dimethylphenyl)azo]-2- naphthalenol	Aniline	3[(b)]	30
1-[(2,4-Dimethylphenyl)azo]-2- naphthalenol	2,4-dimethylamine	3	31
Methyl Orange, Orange II, Ponceau BS, Ponceau S	4-aminobenzenesulfonic acid	-[(c)]	32,33
Para Red	p-nitroaniline	-	
1-[[2-Methyl-4-[(2- methylphenyl)azo]phenyl] azo]-2- naphthalenol	o-toluidine	1[(d)]	31
Methyl Orange, Methyl Red	N,N-dimethyl-p-phenylenediamine	-	32,33
1-(4-Phenylazophenylazo)-2- naphthalenol	p-phenylenediamine	3	34,35
Ponceau BS, Ponceau S	2,5-Diaminobenzene sulfonic acid	-	31
Acid Orange 8, Acid Red 88, Orange II, Para Red, Ponceau BS, 1- phenylazo-2-naphthol, 1-[(2,4- dimethylphenyl)azo]-2-naphthalenol, 1-(4-phenylazophenylazo)-2- nahpthalenol, 1-[[2-methyl-4-[(2- methylphenyl)azo]phenyl] azo]-2- naphthalenol	1-amino-2-naphthol	-	36
Congo Red	Benzidine	1	29
Acid Orange 63	Benzidine-3-sulfonic acid	-	37

(a) Classified by the International Agency for Research on Cancer (IARC) monographs, (b) Not classifiable as to its carcinogenicity to humans, (c) Not in the classification list of IARC, (d) Carcinogenic to human.

and *Verrucomicrobia* phyla[24]. The human intestinal microbiota have essential roles in the digestion, vitamin synthesis, epithelial development, pathogen resistance, and immune responses. Host immune relationship with intestinal microbiota is emerging area, and when the colonization barrier of microorganism disrupted, several types of diseases, including Inflammatory Bowel Disease (IBD) to the host has been seen[25]. Intestinal microbiota has potential effects on liver metabolic activity by influencing gene expression which results in altering the xenobiotic response of liver to different xenobiotics including drugs.

It is very difficult to study of microbial ecology of the human gastrointestinal tract, as it is very difficult to culture them *in vitro* in the laboratory, but modern molecular methods like small subunit rRNA, gene sequencing, and metabolomics have been used to study the intestinal bacteria especially in the study of host-microbe interactions[26]. Human intestinal bacteria degrade various xenobiotics and some of them involve in genotoxicity and carcinogenicity. Azo FCs converted into aromatic amine and less toxic organic intermediate. Most of bio-transformed products of azo FCs produce oxidative stress through free radical formation[28]. Azo FCs bio-transformed through azo reduction by NAD(P)H azoreductase. There are three different types of azoreductase has been found i.e. flavin dependent NADH preferred azoreductase produced by *Escherichia coli*, *Enterococcus faecalis* and *Pseudomonas aeruginosa*, flavin dependent NADPH preferred azoreductase produced by *Bacillus cereus* OY1-2, *Rhodobacter sphaeroides*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* and flavin free NADPH preferred azoreductase produced by *Xenophilus azovorans* KF46F and *Pigmentiphaga kullae*[29]. Toxic metabolites of azo FCs produced by intestinal bacteria on consumption of azo FCs which are genotoxic, and mutagenic in nature are summarized in the Table 1.

4. Immunotoxic Effects

Adverse effect to the immune system includes suppressed ability to resolve an opportunistic infection, exaggerated responses, tissue weight alteration, or cellular marker alterations[38]. Chronic exposure of azo FCs decreases in body weight in male and female, however spleen and thymus[39] relative weight and titles of IgG anti-BSA has observed decreased significantly only in male[40]. Cultured human lymphocytes when treated with azo FCs showed altered leukogram (WBC, neutrophil, lymphocyte, monocyte, eosinophil and basophil) of blood, which strongly ,suggest leukocytosis in exposed animals,[41] in the meanwhile Hashem *et al.*[39] shows *in vivo* experimentally that oral administration of azo FCs significant decrease in monocyte percentage, and decreased the delayed hypersensitivity and concluded that depressing effect on the cellular but not humoral immune response. The function of the immune system depends largely on interleukins such as IL-1β, IL-6, and IL-10 which are involved in the innate immunity and act as the mediator of the inflammatory response. IL-10 is primarily produced by monocytes with various roles in inflammation and immune regulation of innate has been significantly changed when the chronic dose of azo FCs given to the rats[41,42]. Further, immunophenotypic analysis revealed that azo FCs alter the relative expression of CD3e/CD4/CD8 in T cells and CD19 in B-cells. Consistent with the suppression of T-cell and B-cell responses and altered surface receptor expression also lowered the expression of IFN- and TNF- cytokines which suggests that, they may have immunomodulatory effects[43]. The histological and cytological examinations of spleen revealed that, on oral sub-chronic dose of azo FCs can expand in the medulla with cell density and morphology modification, congestion and dilatation of the trabecular blood vessels, and congestion in the red and white pulps with the formation of lymphoid hyperplasia[40,44]. Different histological observation in lesions induced by FCs in spleen has been shown red pulp haemorrhages, vacuolation, focal hyperplasia of the white pulp, and capsular and parenchymal fibrosis. Whereas the pathological parameters like vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (e-NOS) immune labeling markedly increase in chronic and sub-chronic dose of azo food colourants[41]. Aniline (metabolite of almost azo FCs) exposure results into spleenomegaly, hyperplasia, and fibrosis and tumour formation. Like parent molecules aniline itself enhance oxidative/nitrosative stress and lead to damage in proteins, lipids and DNA subsequently in the spleen. Oxidative stress causes transcriptional up-regulation of fibrogenic/inflammatory factors (cytokines, IL- 1, IL-6 and TNF-α) *via* induction of nuclear factor-kappa B, AP-1 and redox-sensitive transcription factors in aniline treated-rats. The

upstream signalling events through phosphorylation of IκB kinases (IKKα and IKKβ) and mitogen-activated protein kinases (MAPKs) could potentially be the causes of activation of NF-**κB** and AP-1. All of these events could initiate a fibrogenic and/or tumorigenic response in the spleen[45]. Cultured human lymphocytes when treated with azo FCs showed significant decrease in mitotic index. Azo FCs and their metabolites significantly increased the chromosomal aberration, aberrant cells and micronucleus values in cultures which indicate those azo FCs and their metabolites have genotoxic potential on human lymphocyte[46].

5. Liver Toxicity

The liver is the major organ that plays a vital role in regulating various physiological processes in our body and has capacity to detoxify toxic substances. It is the targets for many chemicals, but its protective nature make it safe somehow, but acute and chronic level exposure leads to some extent damage even in failure in functioning[47]. Mechanisms of injury for different chemicals are not always known, but when they are investigated, mitochondrial dysfunctions associated with enhanced oxidative stress, energy shortage, accumulation of triglycerides, and cell death are often seen. The toxicant induced mitochondrial dysfunction can be due to itself or reactive metabolites of toxicants generate through cytochrome P450 mediated metabolism[48,49]. Food colourant induces loss of heterozygosity resulting from gene mutation, chromosomal rearrangement, chromosome breakage, or chromosome loss which is well explained in the toxicity of azo and other FCs in the Somatic Mutation And Recombination Test (SMART) of *Drosophila melanogaster*[50]. Azo FCs disrupts the physiology and caused damage to the liver resulting enhanced oxidative stress[47], enzyme disorders[51], DNA damage, mutagenesis[52,53] and carcinogenesis[54].

Several serum biomarkers such as serum Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) are used to evaluate in chronic liver diseases or in case of toxicity of different xenobiotics. Beside these enzymes ALP, urea, creatinine, etc. are also used as a marker to evaluate the functioning status of liver[55,56]. Azo FCs are also reported for their hepatotoxic activity in chronically exposed rats and mice. Tartrazine; a yellow colour permitted food colourant evaluated for their toxicity in rats, and it have been observed that there was a

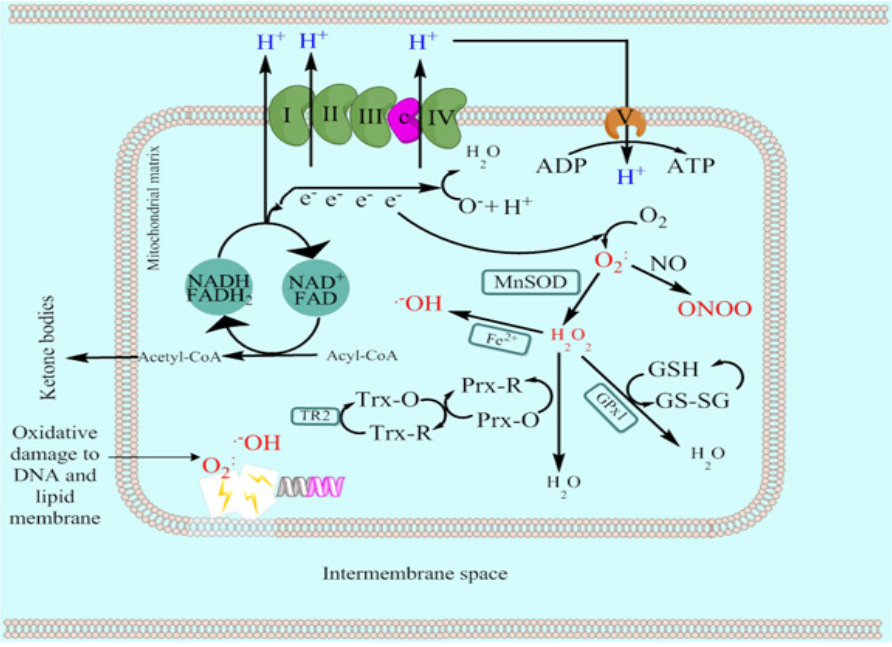


Figure 2. Schematic representation of mitochondrial free radical generation and oxidative damage.

significant increase in ALT, AST, ALP, urea, creatinine in serum[50,57–59]. The study also reported that synthetic colours at low and high dose can change a significant increase in serum glucose concentration, but others reported in contrary to the above findings[57]. Further, experimental animals treated with dye blended water revealed a marked increase in the average liver weight, and severe histological changes in the liver[60]. It was noticed that during the administration period (13 weeks) of colourful foods, rats become more active (hyperactive) and aggressive, and skin of rats shows reddish and irritation after administration of colour for 6 and 12 hours. These results indicate the common cause of chronic urticaria and angioedema in patients induced by the colourants. Administration of both low and high dose of colour food exhibited a significant decrease in liver GSH (a potential antioxidant) content because the synthetic colour caused oxidation of fatty acids in biological membranes lead to increase in free radicals which is able to caused auto-oxidation of the hepatic cells and finally degeneration of hepatocytes. Hence, the consumption of GSH can prevent the cell death by toxic radicals through GSH lead antioxidants resulted hepatic cell degeneration[61,62]. During the β oxidation in the mitochondria, electrons are sequentially transferred to different polypeptide complexes (numbered from I to IV) embedded within the inner membrane. The final transfer of the electrons to oxygen takes place at the level of complex IV, which oxidizes cytochrome c. The flow of electrons within the Mitochondrial Reaction Chain (MRC) is coupled with the extrusion of protons (H+) from the mitochondrial matrix to the inter-membrane space, which creates the mitochondrial trans-membrane potential. When energy is needed (i.e., When ATP levels are low), these protons re-enter the matrix through complex V, thus liberating energy that is used to phosphorylate ADP into ATP. Some of these electrons during the transmission by the mitochondrial complexes react directly with oxygen and form superoxide anion ($O_2^{\cdot-}$) radical and leading to the formation of hydrogen peroxide (H_2O_2) (Figure 2).

The electron reacts with oxygen to form superoxide radical (O_2) which then converted to hydrogen peroxide (H_2O_2) by the superoxide dismutase (SOD). H_2O_2 then react with ferrous iron (Fe2+) to form hydroxide radical (.OH). These ROS damage mDNA, protein and lipid membrane, increasing further mitochondrial ROS production in a vicious cycle. Mitochondria contain several antioxidants enzymes that safely scavenge these ROS. Manganese superoxide dismutase, dismutase O_2 into oxygen and H_2O_2 which is then oxidized into the water by mitochondrial glutathione peroxidase (GPx1), leading to the oxidation of reduced GSH into glutathione disulfide (GSSG), GSSG then reduced back into GSH by GSH reductase. Mitochondrial beta oxidation of fatty acid generates NADH and FADH2, which transfer their electrons to the mitochondrial respiratory chain (MRC), thus regenerating NAD+ and FAD used for beta oxidation cycles.

Manganese superoxide dismutase (a mitochondrial superoxide dismutase enzyme) allows the spontaneous dismutation of the $O_2^{\cdot-}$ into oxygen and H_2O_2, which is then detoxified into water by mitochondrial glutathione (GSH) peroxidase and peroxiredoxins. Alternatively, H_2O_2 reacts with iron to form the highly reactive hydroxyl radical and active myeloperoxidase forms the hypochloride radical[63]. Animal treated with azo FCs revealed a significant increase in the mean MDA concentrations and decrease in the activity of liver hepatic SOD and CAT and GSH levels not only *in vitro*[54] but also *in vivo*[47,58,62]. The unscheduled expression of cyclins in tumor cells may be due to increased and/or continued synthesis or may be impaired degradation during the cell cycle. The continuous presence of cyclins causes activation of their respective partner kinases (cdks), which remain essentially invariable throughout the cell cycle. This, in turn, might lead to unscheduled phosphorylation of a variety of proteins, thus driving the cell through the cycle and abrogating the respective checkpoints. Oral administration of azo food colourants significantly increase in the expression of cyclinD1, cdk4, cyclinB1, and cdc2 were observed in the livers, indicating increased proliferation of cells associated with tumor growth. The hepatic fibrosis shows severe degenerative and inflammatory changes in hepatocytes[54]. Mitochondria-derived ROS activate progression of hepatic fibrotic responses, with a significant up regulation of the levels of the collagen 1α, TGF-β1, and fibronectin mRNAs. The fibrosis consistent with the immunohistochemical localization of collagen 1α and TGF-β1 exposed to FCs[58]. *In vitro* HepG2 cell line High Content Analysis (HCA) indicated azo food colourants could significantly decrease cell number and mitochondrial membrane potential,

accompanied by an increase in the ratio of dead/live cells, activated caspase-3/-7, and increase in intracellular calcium levels and DNA damage[64]. Whereas study also reported that, when rat treated with azo FCs revealed significant increase in tailed nuclei, tail moment, DNA% in the tail, and tail length and the histopathological examination of liver affirmed destructive and degenerative changes[59].

6. Kidney Toxicity

Creatinine estimation is a valuable index of kidney function and it can alter when azo FCs administered to the animal at chronic level[59]. Significant biochemical parameters specially mitochondrial functioning relevant like GSH, MDA, SOD and catalase etc. can change and affect the function of kidney when azo FCs at sub-chronic and chronic level exposure to the animals[47,51,62]. It has been found that immunoreactivity of proliferating cell nuclear antigen in the nuclei of the cells of the renal cortex and medulla was strong in the tartrazine-treated groups compared to the control group[65]. Kidney function can impaired when azo FCs given to the rat, and it shows an increase in the mRNA levels, up-regulation of collagen 1-α, TGFβ-1, TNF-α gene, KIM-1 gene and fibronectin with down-regulation of Casp-9 gene[58,66]. When rat treated with metanil yellow (a non-permitted food colourant), it shows tissue damage through free radical mediated lipid peroxidation and the slices of the kidney showed vacuolization of epithelial lining tubules and endothelial linings and hydropic degeneration of the glomerular and tubular cells [47]. Histomorphological examination of kidney of rats shows that there is hypertrophy of the glomeruli in relation to the size of Bowman's capsule with marked thickening of the glomerular tuft basement membrane and segmented glomeruli has been detected[59,67]. FCs can induce fibrosis, apoptosis, and histopathological perturbations and results can be reflected in the form of periportal fibrosis with tubular necrosis, shrunken glomeruli, and interstitial fibrous tissue proliferation[58].

7. Reproductive Toxicity

Administration of azo FCs can affect in both male and female reproductive organ and system. Earlier studies have been reported changes in testes weight, but the association of histological changes was not very clear[67]. On sub-chronic level administration of food colourants either with food or in water to rodent revealed gain in body weight, but absolute and relative testis, epididymis and seminal vesicles weights did not change. However, sperm count has been decreased and sperm abnormalities increased[68]. In several developmental studies in the F0 generation to nine weeks of age in the F1generation in which azo FCs administered to mice has shown that few adverse effects on litter size, weight, or sex ratio. The survival index during the lactation period significantly lower in the middle-dose group than in controls in each sex and two dams of the middle-dose animals showed underdeveloped mammary glands, which has been observed as underdeveloped mammillae[69–73]. Biochemical markers for functioning of female reproductive organ of rats has showed that significant decrease in serum follicle stimulating hormone (FSH), luteinizing hormone (LH), and estradiol levels when rats were treated with azo FCs at chronic level. Also there is a change in the number and duration of estrous cycle along with prominent cyto-architectural changes in the cellular characteristics of vaginal smear of component phases of estrous cycle with dose duration-dependent manner[74]. In *In vitro* study on Chinese Hamster Ovary (CHO-K1) cell line confirmed that the atrazine decreases ovarian cell proliferation[75]. A significant down regulation of the glial cell derived neurotrophic factor (GDNF), tyrosine kinase receptor (c-KIT), follicle stimulating hormone receptor (FSHR), A kinase anchor protein 3 (PRKA3), spermatogenesis associated 7 (Spata7), Stage-Specific Embryonic Antigen-1 (SSEA1) genes and testicular expression level of biomarkers of testin has been reported in rats supplemented with the azo FCs in dose dependent manner[76]. Prenatal administration of tartrazine to the pregnant rat shows dark brown pigmentation due to hemosiderin in the splenic parenchyma in the fetuses and other morphological deformities which suggest that tartrazine has embryotoxic and teratogenic potentials in rats[77].

8. Neurotoxic Effects

Artificial food colourings have long been suggested to adversely affect the animal behaviours, especially overactive, impulsive and inattentive behaviour (i.e.,

Hyperactivity). Ben Feingold made his initial claims of the detrimental effect of synthetic FCs on childhood behaviour more than 35 years ago and hypothesized that hyperactivity might be a child's adverse reaction to food additives include synthetic colours, since then different kinds of synthetic FCs including azo food colours have been studied separately or as a mixture for behavioural changes[78–80]. In this regard Tanaka and his group give an extensive scientific experimental contribution for analysis of behavioural alterations on exposure of azo FCs[71,73]. On experimental study it has been observed that, azo FCs can elicit adverse effects and can alter biochemical markers (MDA, GSH, SOD and catalase) and neurotransmitters (gamma amino butyric acid, dopamine, and serotonin) in the brain even at prescribed ADI levels[4,81,82]. On neurological safety aspect, *In vitro* pathological study on mouse NB2a neuroblastoma cell line revealed that neurite inhibited with the involvement of N-methyl-D-aspartate (NMDA) receptors[83]. Additionally, the high dose of azo FCs can induce a reduction in the volume of medial prefrontal cortex and its subdivision, and number of neurons, and glial cells as well as the mushroom and thin spines per dendrite length declined with numerous apoptotic cells[81,84]. On histological examination it has been observed that when rats administered azo FCs, numerous pyknotic nuclei in the cerebral cortex, neuronal damage in the cerebrum, medulla and degenerative Purkinje cells with morphologic abnormalities in cellular architectures[65,82]. Prenatal exposure of azo food colourants to the offspring results in a characteristic motor activity and anxiety-like behavioural changes which indicates that development and aging can be vulnerable to those periods in life of offspring[85].

9. Conclusions

Azo FCs are the major volume of the total volume of colourants used in the food industry, off course they have several advantages over the natural and other synthetic FCs but these advantages cannot be compromised with the health and other environmental issues. Their uses are the subject of regulating organization with several aspects like ADI, illegal uses of banned FCs, health consequences, etc., but on the ground level most of the rules and regulations are not followed especially in the developing countries. Importantly, new studies demonstrating that children may actually consume more coloured foods than expected e.g., in 1950 the amount of FCs used to be 12 mg/capita/day which have been raised to 62 mg/capita/day in 2010 in the USA. Several preclinical and some clinical studies reported the effects of azo food colourants, unfortunately these studies have severe limitations; hence it is impossible to draw a clear conclusion on the matter. Although the pharmacological properties and toxicity of food colourants has not yet been exploited, as new and possible useful pharmacological mechanisms of the routinely used colourants should be carried out with modern methodological approaches. Therefore, systematic studies should be performed in order to elucidate pharmacological, development and other effects that various azo colourants or their mixtures may have.

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