Special Article

NUTRITION REVIEWS

Mechanisms of behavioral, atopic, and other reactions to artificial food colors in children

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This review examines the research on mechanisms by which artificial food colors (AFCs) and common foods may cause behavioral changes in children with and without attention-deficit/hyperactivity disorder (ADHD). Children with ADHD show excess inattention, impulsivity, and hyperactivity. Studies have shown that a subgroup of children (with or without ADHD) react adversely to challenges with AFCs. Many early studies found few children who reacted to challenges with 20–40 mg of AFCs. However, studies using at least 50 mg of AFCs showed a greater percentage of children who reacted to the challenge. Three types of potential mechanisms are explored: toxicological, antinutritional, and hypersensitivity. Suggestions for future studies in animals and/or children include dose studies as well as studies to determine the effects of AFCs on the immune system, the intestinal mucosa, and nutrient absorption. Given the potential negative behavioral effects of AFCs, it is important to determine why some children may be more sensitive to AFCs than others and to identify the tolerable upper limits of exposure for children in general and for children at high risk.

INTRODUCTION

In the 1970s and early 1980s, Benjamin Feingold, Chief Emeritus, Department of Allergy, Kaiser-Permanente Medical Center, San Francisco, Calif., proposed that artificial food colors (AFCs), artificial flavors, preservatives, and natural foods containing salicylates (many fruits, a few vegetables, and spices) were common causes of hyperactivity or hyperkinesis.[1–4] By 1980, the terminology "hyperactivity" or "hyperkinesis" was changed to "attention-deficit disorder" (ADD) to describe children who were inattentive but not hyperactive and "attention-deficit/hyperactivity disorder" (ADHD) to describe children who were hyperactive, impulsive, and inattentive. Over the next 35 years, many research groups tested Feingold's hypotheses. Two studies of the Kaiser-Permanente or "Feingold" diet per se versus a sham diet showed that only a small subgroup of children showed improved behavior on the Kaiser-Permanente diet.[5,6]

Subsequently, research teams focused on the effects of AFCs alone on behavior in children diagnosed with hyperkinesis or ADHD. They used dye-challenge studies in which placebo or AFCs were administered as a single bolus dose, after which behavior was monitored for a defined period, or were given consecutively, also followed by behavior evaluations.[6–13] These studies suggested a small subgroup of children who showed significant negative behavioral reactions to the AFCs, but not to the placebo. Five of these studies used low doses of the mixed dyes – 26–35 mg – with only a few children reacting to the challenge.[6,7,9,10,14] Four other studies used larger doses – 50–150 mg – of AFCs, with a greater proportion of

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children reacting to the dyes.[12,13,15,16] Younger children seemed more responsive to the AFCs than older children, and symptoms included irritability and sleep problems in addition to inattention, impulsivity, and hyperactivity.[10,13] Feingold's diet, challenge studies with AFCs in children with hyperkinesis or ADHD, and restrictive diets have been reviewed by Stevens et al.,[17] Arnold et al.,[18] Kanarek,[19] Weiss,[20] and Millichap and Yee.[21]

In addition to these food-dye challenge studies, researchers used oligoantigenic ("few foods") and other elimination diets to test what food additives and common foods might trigger adverse behavioral changes.[22–28] For example, Egger et al.[23] found that many hyperactive children reacted not only to AFCs and preservatives (79% of those tested) but also to many common foods, including milk (64% of those tested), chocolate (59% of those tested), and wheat (49% of those tested). Of those children reacting to AFCs and preservatives, no child reacted to AFCs alone; rather, combination with at least two common foods was required.

In 2004, Schab and Trinh[29] performed a meta-analysis to test whether artificial food colors contribute to the symptoms of hyperactivity in children diagnosed with ADHD as measured by parent rating scales. After analyzing the data from 15 studies, the authors concluded that the hypothesis that AFCs promote hyperactivity in a subgroup of children with ADHD was strongly supported. In 2012, Nigg et al.[30] published a meta-analysis of studies related to ADHD-type symptoms, restrictive diets, and AFCs. They used 24 publications that met their inclusion criteria: the participants were children or adolescents; the study design was double-blind, placebo-controlled crossover; and the study used one or more AFCs as challenge materials and provided information to allow calculation of effect sizes. Ten additional studies that used restrictive diets were analyzed separately. The authors estimated that as many as 33% of children with ADHD may benefit from dietary restrictions and 8% may react to AFCs. The effects of AFCs may not be limited to children with ADHD. In separate studies, Bateman et al.[31] and McCann et al.[32] reported that some children within two general populations of children unselected for ADHD experienced adverse behavioral reactions (hyperactivity) to a mixture of AFCs and sodium benzoate, a preservative, in double-blind, placebo-controlled challenges of the additives compared with placebo. These studies suggest that reactions to AFCs and/or sodium benzoate are not limited to children with ADHD but also occur in the general population. Stevenson et al.[33] identified (using the McCann sample) three genetic markers for susceptibility to the dye mixture: two were histamine genes and one was a dopamine gene. The susceptible allele profile for one of the genes was present in 60% of the sample of unselected children. Therefore, AFCs and/or sodium benzoate may be a problem for substantial subpopulations of children, not necessarily just those with ADHD-type symptoms.

Reactions to AFCs and common foods also occur in some individuals diagnosed with asthma,[34–36] eczema,[34,36,37] chronic urticaria,[34,36,38,39] and migraine.[40–42] The mechanisms by which artificial colors, flavors, preservatives, and/or common foods provoke ADHD-type symptoms and other physical symptoms are not known. This review will explore potential toxicological, antinutritional, and hypersensitivity factors that may help account for reactions to AFCs.

EXPOSURE TO ARTIFICIAL FOOD COLORS

Nine AFCs are currently approved for use in foods in the United States by the Food and Drug Administration (FDA): US FD&C Blue #1 (Brilliant Blue), Blue #2 (Indigotine), Yellow #5 (Tartrazine), Yellow #6 (Sunset Yellow), Green #3 (Fast Green), Red # 3 (Erythrosine), and Red #40 (Allura Red) (Table 1). Citrus Red #2 is only allowed to be used to color orange skins. Orange B is only allowed in hot dog and sausage casings but is no longer used by manufacturers. These dyes are all derived from petroleum. Azo dyes are the most common food dyes and contain one or more azo groups (-N = N-) as part of their chemical structures. This group does not occur naturally in foods. FD&C Red #40, FD&C Yellow #5, and FD&C Yellow #6 are azo dyes and are commonly used in foods consumed by children in the United States. The characteristics of the dye depend on the number and position of the azo groups, the nature of the aromatic nucleus, and the nature and position of substituents such as sulfonates and hydroxyl groups. The charged sulfonate groups on Yellow #5 and Yellow #6 make absorption as well as enzymatic metabolism more difficult.[43] Red #3 is a water-soluble xanthene dye containing four iodine atoms but no azo group. In 1990, Red #3 was banned by the FDA from cosmetics and externally applied drugs because it was shown to cause thyroid tumors in animals.[44] However, it is still allowed in foods, and small amounts (1 mg/capita/day is certified by the FDA) are used to color maraschino cherries, sausage casings, baked goods, and candies. Two triphenylmethane dyes are also approved for use in the United States: Blue #1 and Green #3. Blue #2 is a sulfonated indigo dye. Besides these, several other dyes are permitted to be added only to drugs and/or cosmetics in the United States. Some AFCs are not permitted at all because they are mutagenic and/or carcinogenic in laboratory animals. Lakes that are certified AFCs bound to aluminum are also commonly found in many processed foods, drugs, and cosmetics. The pure dye content of a lake is about 10–40%. Lakes are not soluble and tint by dispersion.

Table 1 **Artificial food colors allowed in the United States by the Food & Drug Administration, along with their acceptable daily intakes and their metabolites.**

FD&C colors	Common name	Type of chemical	Shade	ADI[a] (mg/kg/day)	ADI[a] for 30-kg child	Metabolites in rats and/or humans
Blue #1	Brilliant Blue	Triphenylmethane	Blue	6	180	Unidentified metabolite
Blue #2	Indigotine	Sulfonated indigo	Dark blue	2.5	75	5-sulfoanthranilic acid
Green #3	Fast Green	Triphenylmethane	Blue-green	2.5	75	
Yellow #5	Tartrazine	Azo	Yellow	7.5	225	Sulfanilic acid Aminopyrazalone
Yellow #6	Sunset Yellow	Azo	Orange	3.75	112.5	Sulfanilic acid 1-amino-2-naphthol-6-sulfonic acid p-acetamidobenzene-sulfonic acid
Citrus Red #2[b]	Citrus Red	Azo	Orange			1-amino-2-naphthyl sulfate
Red #3	Erythrosine	Xanthene	Pink	2.5	75	Deiodinated products
Red #40	Allura Red	Azo	Red	7	210	Cresidine-4-sulfonic acid 1-amino-2-naphthol-6-sulfonic acid
Orange B[c]	Orange B					Naphthionic acid

[a] Acceptable daily intake (ADI) represents the amount, in mg/kg of body weight per day, that can be ingested without appreciable health risk.
[b] Only permitted as a dye for orange skins.
[c] Only permitted for use in hot dog and sausage casings, but no batches have been certified for at least 10 years.

Batches of manufactured AFCs must first be certified by the FDA, which tests them for purity and allowable levels of contaminants such as lead, mercury, and benzidine. Based on data obtained from the FDA, the amounts of AFCs certified in the United States by the FDA has increased fivefold, from 12 mg/capita/day in 1955 to 62 mg/capita/day in 2010 (unpublished data obtained from FDA using the Freedom of Information Act) (Figure 1). Red #40 accounts for 40% of the dyes certified by the FDA, Yellow #5 27%, Yellow #6 24%, and Blue #1, Blue #2, Red #3, and Green #3 4% or less each. The Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives is responsible for assessing health risks associated with additives used worldwide and has published an Acceptable Daily Intake (ADI) for each food color, which is the amount, based on research, that can be ingested daily in the diet without appreciable health risks (Table 1). However, different countries allow different dyes in their food supply. For example, the United States permits the use of Yellow #5 but not Quinoline Yellow, while the United Kingdom bans Yellow #5 but allows Quinoline Yellow. Canada allows amaranth, but the United States does not. In the United Kingdom, six colors were subject to a "voluntary phase out" by the end of 2009: Yellow #5, Quinoline Yellow, Yellow #6, carmoisine, Ponceau Red, and Red #40, while the European Union now requires a warning food label if any of these same six AFCs are present, stating "this food may have an adverse effect on activity and attention in children."

The dosages studied as test concentrations in children range from 26 mg to over 100 mg[17] for either a single or a combination dose of AFCs. Per capita amounts of AFCs certified for consumption in the United States currently total 62 mg/day, with Red #40, Yellow #5, and Yellow #6 constituting over 90% of that total. There are no current studies that provide the per capita consumption of AFCs among children in the United States. Studies of countries with Western diets have shown that the per capita consumption of these AFCs for Australian children 2–5 years old was slightly lower than the population mean for Australia, while it was slightly higher than the population mean for children 6–12 years old.[45] It should be noted that the Australian data indicate that per capita consumption of AFCs is much lower than the per capita amounts certified in the United States and is lower than the study dosages. A study of Irish children also showed a lower per capita consumption of these AFCs for children relative to the per capita amounts certified for consumption in the United States.[46] This study also showed that only a small number of Irish children exceeded either of the dosages used in the studies by Bateman et al.[31] and McCann et al.[32]

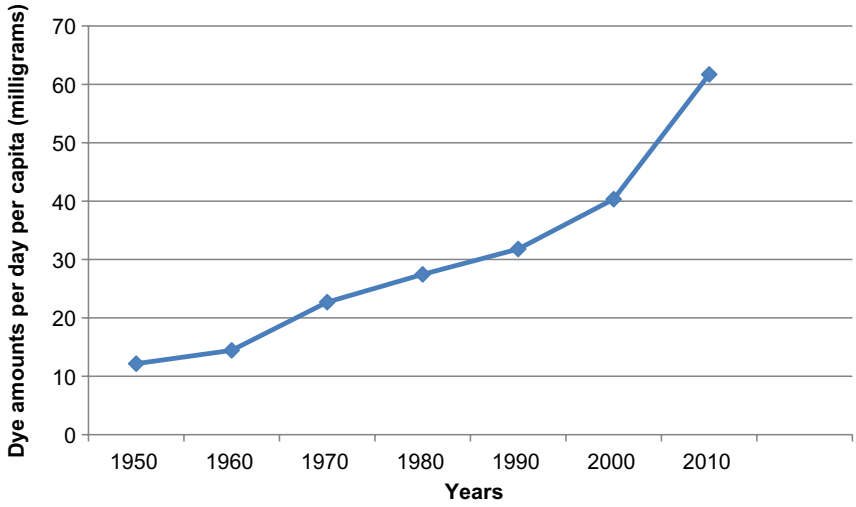

Figure 1 **Per capita per day certification of food dyes (in mg) between 1950 and 2010, as certified by the US Food and Drug Administration.**

In 1991, Marmion[47] estimated the amounts of AFCs in common groups of foods in the United States. He reported that AFCs averaged 100 ppm for candy and confections, 75 ppm for beverages, 140 ppm for dessert powders, 350 ppm for cereals, and 200 ppm for snack foods. Other countries have reported the amounts of AFCs in specific foods in their food supply, but similar data have not been reported for the United States. Some subpopulations may have a greater exposure to AFCs. For example, obese children consume more soft drinks and other sweetened beverages than children of normal weight.[48] In the United States, the prevalence of obesity tripled between 1971 and 2002, while the consumption of sweetened soft drinks also tripled during the same time period.[49] Some soft drinks, sports beverages, and fruit drinks are dyed with AFCs. Cola drinks are dyed with caramel color that is made by heating carbohydrates to a high temperature. The result of this process, Maillard browning, is the formation of advanced-glycation end products, which are harmful in large amounts,[50–52] but a discussion of this topic is beyond the scope of this paper. Most soft drinks are sweetened with high-fructose corn syrup, which is seven times more likely than glucose to form advanced-glycation end products.[50]

ABSORPTION OF ARTIFICIAL FOOD COLORS

Several studies in laboratory animals and one in humans have shown that, generally, only very small amounts of AFCs are absorbed intact and excreted in the urine (Table 2). Like many other low-molecular-weight chemicals, small amounts of azo dyes can be absorbed sublingually and buccally by passive diffusion in humans.[53,54] Yellow #5 and other azo dyes also can be absorbed in small amounts (≤5% for Yellow #5) through the skin, where they undergo azo reduction.[55] For azo dyes, unchanged dye in the urine ranges from none detected to 2–4% of the dose. When 10 mg of radiolabeled Yellow #5 was given to rats, 8% was recovered in urine and 90% in feces.[56] Honohan et al.[57] administered ^{14}C-Yellow #5 to rats and reported 4% excreted in urine and 87.3% in the feces. They also fed ^{14}C-Yellow #6 to rats, reporting 8.5% excretion in urine and 94.5% in the feces. These results included radioactivity from both intact dyes and their radioactive metabolites.

At least four factors may influence absorption of AFCs: intestinal motility, dye characteristics, pH, and intestinal integrity. Hess and Fitzhugh[58] reported that the amount of triphenylmethane colors recovered in bile was in part dependent on the speed with which the dyes traveled through the intestinal tract, thus indicating the role of intestinal motility. The slower the speed, the more dye was absorbed and secreted into bile. Webb et al.[59] studied various xanthene dyes, including Red #3. These dyes are halogenated forms of fluorescein. They noted that the amount of dye excreted in the urine and bile depended upon the degree of halogenation of fluorescein, indicating the influence of physical and chemical characteristics of the dyes. The more halogenation (Red #3 has 4 iodine atoms, while fluorescein has none), the more dye was recovered in bile in the gallbladder, but less dye was recovered in the urine. Those dyes with several halogen atoms, like Red #3, were excreted in significantly shorter transit times than was fluorescein, which has no halogen atoms. Webb et al.[59] also reported that the stability of xanthene dyes in warm acid increased with increased halogenations, indicating the influence of pH on absorption. Studies of absorption of AFCs from the intestinal tracts of

Table 2 **Absorption and excretion of oral doses of artificial food colors in laboratory animals and humans.**

Type of dye	Dye	Reference	Animals/humans	Oral dose (mg)	Recovered intact dye (% of dose)			Metabolites in urine (% of dose)	Recovered radioactivity (%)	
					Urine (%)	Feces (%)	Bile (%)		Urine (%)	Feces (%)
Azo	Tartrazine, ^{14}C-tartrazine	Honohan et al. (1977)[57]	Rats	10	ND	87.3	Trace	21.4	4	87.3
	^{14}C-Sunset Yellow	Honohan et al. (1977)[57]	Rats	2–25	0.3	94.5	1.5	37.4	8.5	94.5
	Tartrazine	Jones et al. (1964)[87]	Rats	5	ND			58.9–64.6		
			Humans	100	ND			87–106		
	^{35}S-tartrazine	Ryan et al. (1969)[56]	Rats	10					8	90
Xanthene	Erythrosine	Daniel (1962)[84]	Rats	64–156	ND	55–72	0.4–1.7			
Triphenylmethane	^{14}C-blue #1	Brown et al. (1980)[85]	Rats	0.27					0.22	91.14
	Blue #1	Hess & Fitzhugh (1955)[58]	Rats	200	ND	96				
			Dogs	200			0.7–2.8			
	Green #3	Hess & Fitzhugh (1955)[58]	Rats	200	ND	94				
			Dogs	200			1.7–2.2			
Sulfonated indigo	^{35}S-blue #2	Lethco & Webb (1966)[86]	Rats	5					1.61	60.2
				25					1.65	64.6
				50					2.10	77.9

Abbreviations: ND, none detected.

children have not been reported. Whether food dyes are absorbed intact or only as metabolites is not known. One hypothesis is that abnormal intestinal permeability ("leaky gut"), which allows entry of some normally excluded harmful molecules into intestinal cells, might increase absorption of AFCs in some children. Increased intestinal permeability can occur following the use of antibiotics[60] or nonsteroidal anti-inflammatory drugs[61] or in individuals with celiac disease, Crohn's disease, food allergies, cow's milk intolerance, excessive fructose intake,[62] exposure to environmental toxins, and low-fiber diets.[63] Stevens et al.[64] reported that antibiotic usage since infancy was significantly greater in 53 children with ADHD-type symptoms than in 43 controls ($P < 0.02$). Celiac disease is marked by inflammation in the small intestine due to gluten intake, causing atrophy of villi in the jejunum and leading to malabsorption of important nutrients and absorption of molecules normally excluded. Although the incidence of celiac disease has not been studied in children with ADHD, ADHD-type symptoms have been reported in children and adults with celiac disease.[65–67] Niederhofer and Pittschieler[68] assessed ADHD symptoms in 78 patients with celiac disease aged 3–57 years (average age, 19.3 years) before the start of a gluten-free diet using patient or parent behavior rating scales. Six months after starting the diet, ADHD-type symptoms, such as inattention, impulsivity, and overall symptom score, were significantly improved ($P < 0.001$). Whether children with celiac disease absorb more AFCs has not been reported.

Excessive intake of fructose is another factor that may affect intestinal permeability.[62] In the United States, the intake of high-fructose corn syrup has greatly increased from very low levels in the 1970s to more than 100 g/day/person in 2007.[69] Nondiet soft drinks are highly sweetened with high-fructose corn syrup, and a 20-ounce soda pop may contain more than 32 g of fructose.[50] Children may have a limited ability to absorb excessive fructose.[70] Chronic excessive intake can lead to small intestinal bacteria overgrowth and increased intestinal permeability.[71] Importantly, foods and beverages sweetened with high-fructose corn syrup often contain large amounts of AFCs, potentially leading to greater absorption of these dyes.

Food allergy is another cause of increased permeability. Andre et al.[72] measured the absorption of mannitol (a marker of normal monomer passage) and lactulose (a marker of macromolecule exclusion) in 15 healthy subjects and 20 subjects with food allergies controlled by dietary exclusion. There were no significant differences at baseline. However, when an offending food was consumed, there was a significantly increased absorption of lactulose ($P < 0.001$) over the fasting state, and the lactulose/mannitol ratio doubled, suggesting an increase

in intestinal permeability. Increased intestinal permeability has also been reported in patients with other allergic disorders, including asthma,[73,74] eczema,[75–78] and chronic urticaria.[79]

METABOLISM AND EXCRETION OF ARTIFICIAL FOOD COLORS AND/OR METABOLITES

Most azo dyes are metabolized in the gut by azo reductases from anaerobic intestinal bacteria, yielding sulfanilic acid and metabolites such as N,N-dimethyl-p-phenylenediamine, 1-amino-2-naphthol, m-xylidine-6-sulfonic acid, cresidine-sulfonic acid, sodium-naphthionate, and R-amino salt[80–82] (Table 1). At least 10 distinct common intestinal microorganisms produce azo reductases.[80,83] In the liver, azo dyes are reduced to aromatic amines by azo reductases.[80]

Metabolism of other types of dyes has also been studied in rats. Daniel[84] reported that Red #3 is excreted largely in the feces (50–70%), with less than 1% excreted in bile, and he hypothesized that some of the rest was metabolized in tissues. Hess and Fitzhugh[58] reported that 10 mg of a 200-mg dose of Blue #1, a triphenylmethane dye, was absorbed from the gastrointestinal tract in rats. Brown et al.[85] measured the absorption of Blue #1 in the intestines of rats given a single dose (0.27 mg) of ^{14}C-labeled dye. Approximately 91.7% of the dye was recovered unchanged: 0.2% was recovered in the urine, 91% in feces, and very little in organs or bile. Biliary excretion in bile-cannulated animals averaged 1.32% of the dose. Lethco and Webb[86] studied the metabolism of Blue #2 in rats and reported that small amounts of dye were absorbed intact and excreted in urine, but the metabolite, 5-sulfoanthranilic acid, was more readily absorbed when given orally, with about 24% excreted in the urine.

While only small amounts of intact AFCs are excreted in urine, large amounts of dyes are excreted in the feces. However, some metabolites can be absorbed into the general circulation and excreted in either urine or the feces. Jones et al.[87] gave four healthy men 100 mg of Yellow #5 in a capsule. No unchanged dye was found in the urine, but amounts of free and conjugated sulfanilic acid in urine were equivalent to the dose of Yellow #5.

BLOOD–BRAIN BARRIER AND ARTIFICIAL FOOD COLORS

There is little evidence to suggest that most intact dyes cross the blood–brain barrier. One exception is Blue #1. The ability of this dye to cross the blood–brain barrier has been used therapeutically by intravenously administering a Blue #1 derivative to ameliorate neurodegeneration in certain animal models; the dye blocks P2X7 receptors, which are significantly upregulated in several neurodegenerative diseases.[88,89] Brain uptake of Red #3 has been studied by Levitan et al.[90] They reported that Red #3 can cross the blood–brain barrier when not complexed, but it usually binds to plasma proteins, which restricts passage of the Red #3-protein complex. The effects of sulfanilic acid also have been studied in rat pups by injecting them daily with intraperitoneal sulfanilic acid.[91,92] Administration of sulfanilic acid was followed by hyperactivity and impaired shock performance. The authors hypothesized that sulfanilic acid may cross the blood–brain barrier and may be one of the causative agents of behavioral changes in rats given azo AFCs.

POTENTIAL MECHANISMS

How might AFCs cause behavioral and physical symptoms in some children? Three potential mechanisms are discussed.

Toxicity

Toxicity is the degree to which a substance can harm humans or animals. These substances include chemicals, drugs, pollutants, heavy metals, organic solvents, pesticides, and food additives. Even substances that normally support life, like oxygen and water, may be toxic if the intake is high enough, so dosage is important. Toxic chemicals can affect any organ of the body, including the brain.

Three studies measured the effects of AFCs in male albino rats on four markers of physiological toxicity: growth impairment, liver enzymes, kidney dysfunction, and blood cells (see Table 3).[93–95] The doses used included a very large amount (0.8 g/kg bw [body weight]),[93] a lower dose range (70–124 mg/kg diet),[94] and one low dose and one high dose (15 mg/kg bw and 500 mg/kg bw, respectively).[95] The AFCs used also differed in the studies: a mixture of Yellow #6, Yellow #5, carmoisine, and Blue #1[93]; Blue #1, carmoisine, and Yellow #5[94]; and Yellow #5 and carmoisine.[95] The experimental time periods ranged from 30 days to 60 days. Weight gain was suppressed in two studies.[94,95] In general, the larger doses (70 mg/kg or greater) significantly increased indicators of liver stress: aspartate transaminase, alanine transaminase, and alkaline phosphatase. Markers of kidney function (total protein, albumin, and globulin) were significantly increased, and there were abnormalities in counts of both red and white blood cells. Most importantly, the study using just 15 mg/kg bw after 30 days showed significantly higher levels of serum alkaline phosphatase, total protein, albumin, urea, and creatinine than controls, and superoxide dismutase and body weight were significantly lower.[95]

Table 3 **Toxicological studies of the effects of artificial food colors on rats and mice, brain tissue, and cell cultures.**

Reference	AFCs studied	Animals	Dosage	Length of study	Physiological changes	Neurochemical changes	Behavioral toxicity
Aboel-Zahab et al. (1997)[93]	Mixture: Yellow #5, Yellow #6, Blue #1, carmoisine[a]	Male albino rats; 30 per group	0.8 g/kg bw	60 days	↑ Liver enzymes ↓ RBC, neutrophils ↑ Eosinophils, lymphocytes		
Abd El-Wahab & El-Deen Moram (2013)[94]	Mixture: Blue #1, carmoisine, Yellow #5	Male albino rats; 10 rats per group	Blue #1: 124 mg/kg diet; carmoisine: 70 mg/kg diet; yellow #6: 75 mg/kg diet	42 days	↓ Body weight ↑ Liver enzymes ↓ Hemoglobin, RBC		
Amin et al. (2010)[95]	Yellow #5	Young male rats	15 mg/kg bw	1 month	↑ ALP, total protein, albumin, urea creatinine		
Gao et al. (2011)[103]	Yellow #5	Male and female rats, mice	175, 350, and 700 mg/kg bw	30 days		↓ Catalase, superoxide dismutase, glutathione peroxidise ↑ Malondialdehyde	↑ Learning, memory impairment, hyperactivity
Shaywitz et al. (1979)[92]	Mixture: Blue #1, Blue #2, Green #3, Red #3, Yellow #5, Yellow #6, Orange B	Rat pups	0, 0.5, 1.0, and 2 mg/kg bw	1 month			↑ Motor activity ↓ Activity habituation
Tanaka et al. (2008)[100]	Yellow #5	Male mice	0.05, 0.015, and 0.45% of diet	3 generations			↓ Motor activity of exploratory behavior
Kamel & El-lethey, (2011)[99]	Yellow #6	Weanling rats	0, 1.0, and 2.5% of diet	16 weeks			↑ Motor activity ↑ Anxiety ↑ Depression symptoms
Goldenring et al. (1982)[91]	Sulfanilic acid	Sprague Dawley rats	1 mg/kg bw	1 month		Total brain concentration of dopamine, norepinephrine unchanged	↑ Motor activity ↑ Shock avoidance
Dalal & Poddar (2009)[102]	Red #3	Young male albino rats	10, 100, and 200 mg/kg bw	1 day (single oral dose)		↓ 5-hydroxytryptophan in medulla pons, hypothalamus, hippocampus ↑ Monoamine oxidase, 5 hydroxyindoleacetic acid in hippocampus	↓ Motor activity
Lau et al. (2006)[101]	Blue #1, L-glutamic acid, Quinoline Yellow[a], aspartame	Mouse neuroblastoma cells	Blue #1: 0.05 nM; Quinoline Yellow: 0.5 µM; glutamic acid: 0.5 µM; aspartame: 0.5 µM			↓ Neurite growth	

[a] Not approved for use in foods in the United States.
Abbreviations: ALP, alkaline phosphatase; bw, body weight; RBC, red blood cells.

Although some of the studies discussed above (and below) appear to have used large doses of AFCs, scientists studying drug dosage extrapolation from rats to humans report that drug doses used in rats should be higher than doses used in humans because the intestinal surface area in the rat is much smaller than the intestinal surface area in humans.[96,97] Using an equation derived by Reagan-Shaw et al.[96] and based on body surface area, the ADI for Yellow #5 of 7.5 mg/kg for children translates to 31.25 mg/kg in the rat.

While the previous three studies indicated classic, toxic physiological changes following chronic intake of AFCs, behavioral toxicology is also important. Behavioral toxicology studies the effects of toxins on the behavior and central nervous system in animals and humans along with the amounts required to cause undesirable behavioral effects.[98] Mercury, aluminum, lead, and alcohol are just a few of the well-studied behavioral toxicants. Importantly, behavioral toxicology studies were not considered in arriving at the ADI for each AFC. Future adjustments to ADIs should address this issue.

Toxic behavioral effects of AFCs may differ between species, between different AFCs, and with dose. Shaywitz et al.[92] studied the effects of a mixture of AFCs (Blue #1, Blue #2, Green #3, Red #3, Yellow #5, Yellow #6, and Orange B) given to young rat pups for 1 month at three different doses (0.5, 1.0, and 2 mg/kg bw). There were many measures of behavior, with only a few differences between the intervention groups and the controls: significantly increased motor activity ($P < 0.001$) and a reduction of activity habituation ($P < 0.001$) in those rats receiving the two higher doses of AFCs. There was no clear dose response, but rats receiving the highest dose had the greatest activity and significant effects on activity habituation. Similarly, Kamel and El-lethey[99] reported a study in which drinking water to which Yellow #5 had been added at two different concentrations (1.0% and 2.5% of diet) was given to 45 weanling rats (15 rats per group) for 16 weeks. Rats given Yellow #5 showed hyperactivity, increased anxiety, and depression-like behaviors. Conversely, Tanaka et al.[100] administered Yellow #5 at different doses (0.05, 0.15, and 0.45% of diet) to three generations of mice. Decreased motor activity of exploratory behavior (total distance $P < 0.05$, average distance $P < 0.01$, and average speed $P < 0.01$) were reported in male offspring of the F2 generation at 3 weeks of age.

Lau et al.[101] reported a neurotoxicity study in mouse cells using a mixture of four food additives: Blue #1, L-glutamic acid as in monosodium glutamate, Quinoline Yellow (banned in the United States), and aspartame. The concentrations used in the study were chosen to reflect AFC intake in children. In vitro, researchers induced the growth and differentiation of mice NB2 neuroblastoma cells and measured neurite growth in the presence of the additives. Neurite growth is a physiological process that indicates cellular well-being. Blue #1 (which crosses the blood–brain barrier) had the greatest effect on inhibiting neurite growth, followed in decreasing order by glutamic acid, Quinoline Yellow, and aspartame. Significant synergy for inhibiting neurite growth was also reported with combinations of Blue #1and L-glutamic acid, and Quinoline Yellow and aspartame.

A few studies have looked at both behavior and neurochemical changes that occurred after the administration of AFCs or their metabolites. Goldenring et al.[91] administered 1 mg/kg bw sulfanilic acid (a major metabolite of azo dyes) via intraperitoneal injection to Sprague Dawley rat pups for the first postnatal month. Significant changes in increased activity ($P < 0.005$) and shock escape avoidance ($P < 0.005$) were seen in the group receiving the sulfanilic acid. This showed that this azo dye metabolite could alter behavior. Total brain concentrations of dopamine and norepinephrine were not significantly affected by the sulfanilic acid treatment. Although regional and localized concentrations of neurotransmitters were not measured, they might have been more revealing. In contrast to hyperactive behavior produced by Yellow #5 in rats, other variant biochemical and behavioral changes have been reported in rats given different AFCs. Dalal and Poddar[102] gave young adult male rats a large oral dose of Red #3 (10,100 or 200 mg/kg bw). Motor activity was reduced at 2 hours, and serotonergic activity also decreased in the brain, as indicated by levels of 5-hydroxytryptophan in the medulla pons ($P < 0.01$), the hypothalamus ($P < 0.01$), and the hippocampus ($P < 0.01$). In the brain, 5-hydroxytryptophan is the precursor for serotonin, which regulates mood, appetite, sleep, memory, and learning. Monoamine oxidase A and 5-hydroxyindoleacetic acid increased only in the hippocampus ($P < 0.01$). Monoamine oxidase A is the enzyme that deaminates norepinephrine, epinephrine, serotonin, and dopamine, while 5-hydroxyindoleacetic acid is the main metabolite of serotonin. Gao et al.[103] administered Yellow #5 to mice at three different doses (175, 350, and 700 mg/kg bw) for 30 days. Behavioral tests suggested that the two higher doses significantly affected measures of learning, memory, and hyperactivity and decreased exploratory behavior. Likewise, the researchers administered the same doses to rats for 30 days. The two higher doses significantly affected spontaneous exploratory activity and markers of oxidative stress in the brain: decreases in the activities of catalase, glutathione peroxidase, and superoxide dismutase were reported, as well as an increase in malondialdehyde levels. However, the low dose did not affect behavioral test results or oxidative stress markers.

Tiny amounts of toxic chemicals – two aromatic amines, benzidine and aniline – have been reported in

samples of some AFCs,[104–106] especially Yellow #5 and Yellow #6, and also in foods containing AFCs.[107] Benzidine and other nonsulfonated aromatic amines are present during the manufacture of azo dyes and may be present in both free and combined states in the final food dyes. Lancaster and Lawrence[107,108] measured total nonsulfonated amines in soft drinks and hard candies and reported small measurable amounts of aniline, 1-naphthylamine, and benzidine in some samples but not in others. When azo dyes are ingested, azo reductases in the intestine cleave the azo bond and liberate free aromatic amines that can form reactive oxygen species, leading to increased oxidative stress. Whether these aromatic amines affect behavior in children and what amounts are toxic are not known. Lead, arsenic, and mercury are also found in some batches of dyes, but the FDA limits these to 10 ppm, 3 ppm, and 1 ppm, respectively.

Aluminum lake colors – AFCs adsorbed onto an aluminum hydroxide substrate – are also found in foods, drugs, and cosmetics. In the stomach, the aluminum substrate dissolves, liberating the dye from aluminum.[43] Aluminum itself is known to be a neurotoxin and can be absorbed in the intestine.[109] Animals exposed to chronic aluminum intake show behavioral, neuropathological, and neurochemical changes.[110] Based on data from the 1987 U.S. National Academy of Science Survey and on 1988 data from the International Association of Color Manufacturers, Soni et al.[111] estimated the average per capita daily intake as 2 mg lake (1.1 mg elemental Al) for 1987 and 14.7 mg lake (7.79 mg elemental Al [54.5 mg/week]) for 1998. In 2006, the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives[112] set the provisional tolerable weekly intake of aluminum at 1 mg/kg bw.

Hypersensitivity mechanisms

The behavioral effects of AFCs may be mediated through hypersensitivity mechanisms.[28,113] The term "hypersensitivity" refers to both allergic and nonallergic reactions to AFCs, foods, and inhaled particles.[114] The term "food allergy" refers to immunoglobulin E (IgE)-mediated reactions to foods such as cow's milk, eggs, peanuts, seafood, or wheat elicited by skin prick tests or radioallergosorbent tests (RASTs) for offending foods, with total IgE levels being elevated. Typical symptoms include asthma, allergic rhinitis, eczema, and chronic hives. There are also non-IgE-mediated food and food additive reactions that produce similar symptoms that can be referred to as "nonallergic hypersensitivities." Both IgE-mediated and non-IgE-mediated reactions have been shown to occur in patients with typical allergic symptoms. Whether both of these types of reactions occur in children with AFC-induced behavior changes is not clear. Both involve activation of certain components of the immune system, which can lead to histamine release and increased leukotriene production. Leukotrienes play important roles in allergy and inflammatory disease.

AFCs can be referred to as "haptens" because of their low molecular weight. Common haptens include some drugs, preservatives, cosmetics, and food ingredients. Haptens can elicit an immune response only when they are attached covalently to a large carrier molecule, such as a protein in the gastrointestinal tract.[115] Sensitization to the hapten carrier results in activation of T cells and release of granules and cytokines by mast cells, which trigger typical allergy symptoms and increased IgE levels.

Yellow #5 appears to cause inflammation in the gastric mucosa in both rats and humans. Rats receiving 7.5 mg/kg/day of Yellow #5 for 10 months showed significant increases in lymphocytes ($P < 0.05$) and eosinophils ($P < 0.05$) in the gastric antrum mucosa.[116] Lymphocytes, both T and B cells, and eosinophils are commonly elevated in allergic persons. The authors concluded that these responses to Yellow #5 suggested an immune-type response. The gastric mucosa in humans can also be affected by Yellow #5. Schaubschlager et al.[117] evaluated the effects of Yellow #5 in three patients who had a history of intolerance to food additives that caused migraine, eczema, stomatitis, and nausea. Using an endoscope, they applied 5 mg of Yellow #5 to stomach mucosa. Macroscopically, the researchers observed erythema, swelling, and petechial bleeding, all signs of inflammation. Histologically, they observed degranulation of mast cells. Biochemically, total tissue histamine release was studied before and after application of Yellow #5, and a loss of total tissue histamine was noted. Inflammation can occur in both IgE-mediated allergic reactions and non-IgE-mediated reactions. IgE levels were not reported, so the mechanism of the effects of Yellow #5 on the stomach mucosa is not completely clear.

Pelsser and Buitelaar[118] hypothesized that a nonallergic hypersensitivity disorder can trigger ADHD-type symptoms in some children. This type of response is not IgE mediated but produces reactions and symptoms that share some similarities with immunological reactions: mast cells are activated, cytokines and histamine are released, and the ratios of prostaglandins and leukotrienes are altered by exposure to these hapten-carrier molecules. Behavioral reactions to food dyes may occur independently of atopy, perhaps by a non-IgE-dependent release of histamine from mast cells and basophilic granulocytes.[31,33,118]

Mast cells, basophils, and platelets all secrete histamine, which is stored intracellularly in vesicles until release is stimulated by either allergens or nonallergenic stimuli. When histamine is released, it can bind to recep-

tors on target cells in many tissues. Murdoch et al.[39] measured leukocyte histamine release in response to azo dyes in 18 controls and 12 patients with chronic urticaria. In this in vitro study, concentrations of AFCs added to the leukocytes were low, ranging from 1 ng/mL to 1,000 ng/mL. These amounts were chosen by assuming a daily intake of 100 mg azo dyes and an absorption of 2% followed by subsequent dilution in body fluids. The histamine release after the addition of azo dyes was more than 12.3% of the total cell histamine in 4 of 18 normal subjects and in 2 of 12 of the patients with chronic urticaria. Murdoch et al.[119] also tested the effects of different doses of Yellow #5 on histamine levels in the plasma and urine of 10 healthy adults. Five had a history of atopy, and five did not. When subjects were given 150 mg of Yellow #5, plasma ($P < 0.005$) and urinary ($P < 0.05$) histamine levels increased significantly, yet the subjects showed no allergic symptoms. The authors concluded that a large dose of Yellow #5 – or its metabolites – induced histamine release in some participants. Di Lorenzo et al.[120] also studied the effects of AFCs in vivo on histamine release in patients with chronic urticaria. Those patients who had demonstrated urticaria and/or angioedema in response to one or more food additives, including Yellow #5 and Red #3, experienced symptoms when challenged with a mixture of the additives and excreted increased amounts of urinary methylhistamine ($P < 0.0001$) and leukotriene E4 (LTE_4) ($P < 0.0001$).

Stevenson et al.[33] studied genetic polymorphisms in N-methyl transferase (HNMT), the gene that codes for histamine degradation. McCann et al.[32] recruited 153 3-year-olds and 144 8–9-year-olds from general community samples and challenged them with mixed dyes and sodium benzoate. The children showed significant behavioral changes after ingesting the mixture but not after ingesting the placebo. As part of the study, DNA was collected from each child and analyzed for several genes. Stevenson et al.[33] reported that, based on the DNA samples, the ADHD-type behaviors observed in a subpopulation of children who reacted to the AFCs and sodium benzoate were moderated by histamine degradation gene polymorphisms HNMT T939C and HNMT Thr105lle in 3-year-old and 8–9-year-old children.

What seems to be clear from these studies is that hypersensitivity reactions to foods and food additives occur in both allergic and nonallergic children. IgE-mediated allergies to foods can lead to typical atopic symptoms such as asthma, hay fever, eczema, and hives. Hypersensitivities to foods that are not IgE mediated may also cause the same symptoms and may share part of the same biochemical pathway that leads to these symptoms. AFCs can trigger these same symptoms as well as migraines, seizures, and ADHD-type symptoms, all of which affect the brain. Why these hypersensitivities occur is unclear, but they have been shown to involve components of the immune system.

Nutritional effects of AFCs: interfering with essential nutrient bioavailability

Zinc is a micronutrient essential for over 100 different metalloenzymes and metal–enzyme complexes, many of them in the brain, where zinc is important for both structure and function. Zinc acts as a cofactor in the metabolism of neurotransmitters, prostaglandins, melatonin, and, indirectly, dopamine.[121] Zinc deficiency has been associated with hyperactivity and other behavioral changes in animals.[122–124] Some researchers have reported low zinc status in children with ADHD, but primarily in Middle Eastern countries, where food intakes are different from those in Western countries.[125–127] Researchers in the United States did not find zinc supplementation (15 mg/day zinc glycinate) helpful for children with ADHD,[121] but children were not selected on the basis of low zinc levels.

Research in hyperactive children suggests that Yellow #5 and Yellow #6, given in a single dose, may chelate zinc so that long-term consumption of these dyes may cause chronic zinc depletion. Ward et al.[128] recruited 20 hyperactive boys and 20 age-matched controls. At baseline, the hyperactive children were reported as having lower mean zinc levels than the controls, with significant differences for hair ($P < 0.001$), serum ($P < 0.01$ to <0.05), 24-hour urine specimens ($P < 0.001$), and fingernails ($P < 0.01$). Both the hyperactive and the control groups were each divided into two groups. One group drank a mixed commercial beverage known to contain Yellow #5, and the other group drank a similar orange drink without dyes. After the Yellow #5 challenge, 24-hour urinary zinc was significantly increased (between $P < 0.01$ and $P < 0.05$), while serum zinc decreased nonsignificantly from baseline in the hyperactive children. Zinc levels were unchanged in the controls. In a replication study, Ward[129] administered 50 mg of Yellow #5, Yellow #6, or amaranth to 47 hyperactive children and three placebo groups. Neither the children in the placebo group nor those challenged with amaranth showed changes. Those receiving Yellow #5 or Yellow #6 showed nonsignificantly lower levels of zinc in serum and significantly higher levels in urine ($P < 0.01$) 120 minutes after the dye challenge. Yellow #5 and Yellow #6 appeared to cause a zinc-wasting effect in some hyperactive children. This could be important because of the essential role of zinc as a metalloenzyme in the brain. Additionally, zinc deficiency can lead to damaged microvilli and increased intestinal permeability.[66]

Marginal zinc deficiencies could also lead to oxidative stress. Zinc is a key component of superoxide dismu-

tase of the antioxidant defense system, which catalyzes the conversion of superoxide to oxygen and hydrogen peroxide. Superoxide dismutase also neutralizes the nitric oxide radical.[130] In spontaneously hypertensive rats (SHRs), which have behavior and biochemical abnormalities similar to those of children with ADHD, supplementing zinc in the diet increased copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) activity in erythrocytes, reduced levels of lipid peroxides ($P < 0.001$), and decreased systolic blood pressure after 4 months ($P < 0.001$).[131] Effects of zinc on behavior have not been studied in SHRs.

Yellow #5 may chelate iron as well as zinc. Oner et al.[132] reported that both low zinc and low iron (serum ferritin) levels were associated with higher scores on parent rating scales in 118 Turkish subjects with ADHD. Konofal et al.[133] reported that mean serum ferritin levels were lower in 53 French children with ADHD than in the 27 controls ($P < 0.001$). Eighty-four percent of the children with ADHD versus 18% of the controls had low serum ferritin. The degree to which AFCs chelate iron and other minerals needs further study.

SUMMARY AND FUTURE STUDIES

Many questions remain about the mechanisms by which foods and food additives cause biochemical changes that lead to abnormal changes in behavior in children, though several aspects are known: 1) AFCs can cause adverse behavioral changes in a subgroup of children with ADHD and in a subgroup of the general pediatric population as well. They can also cause behavioral abnormalities in laboratory animals, particularly at high doses. 2) The azo dyes are metabolized in the gut by azo reductases secreted by intestinal bacteria. While the absorption of intact dyes is low, some metabolites of dyes are more readily absorbed. 3) Following challenge with AFCs, histamine is released into the plasma and excreted in the urine in some humans but not in others. 4) It is unclear whether the hypersensitivity reaction is an IgE-mediated reaction, but it may have an immunological component.

Several types of studies are proposed here as a means to learn more about the possible mechanisms by which AFCs provoke behavioral changes in children. Animal studies could be helpful in elucidating the mechanisms. SHRs were bred to be a model for hypertension. In addition to hypertension, SHRs show many of the same symptoms as children with ADHD – inattention, impulsivity, and hyperactivity – and also share some of the same neurochemistry.[134,135] The behavioral changes occur in juvenile SHRs before the onset of hypertension. SHRs have been used extensively to test the effects of drugs on behavior, but there are no reports of the effects of AFCs on either behavior or biochemistry in SHRs. Behavioral tests before and after a challenge with AFCs (singly and mixed) and sulfanilic acid would be appropriate in an SHR model and also in normal rats. The effects of chronically consumed AFCs on absorption and metabolism in the small intestine and on intestinal measures of oxidative stress would also be of interest. Studies investigating the behavior of SHRs as well as their biochemical reactions to AFCs could contribute much toward understanding the mechanisms.

In children with ADHD, much about the absorption of foods and AFCs is still unclear. Intestinal permeability tests at baseline, after eating a diet containing no AFCs for 2 weeks and after challenge with AFCs, could be revealing: Is intestinal permeability in test subjects greater than that in controls before the challenge, and what is the effect of the challenge? A simple permeability test to measure levels of the sugars lactulose and mannitol in urine is easy and noninvasive.

To better understand the relationship between hypersensitivities to dyes and ADHD-type symptoms, a study of the biochemical and behavioral effects of AFCs on children both with and without atopy would be helpful. Baseline IgE, histamine, histamine receptor mRNA, white blood cell counts – especially basophils and eosinophils – urinary IgE, histamine, interleukins, and leukotrienes would be measured. Next, the children would follow a diet free of AFCs, artificial flavors, and preservatives for 2 weeks. The children would then participate in a crossover, double-blind, placebo-controlled trial in which they would consume Yellow #5 or some other AFC for 1 week and a placebo for another week, with a washout week in between. At the end of each week, the same markers measured at baseline would be measured again. Behavioral measures would also be assessed pre and post challenges.

Urine and plasma levels of zinc before and after a single AFC challenge, as Ward[129] reported, could also include measures of other nutrients such as copper and iron, which could bind to AFCs. Plasma zinc is not always sensitive to small changes in zinc status and is affected by recent meals, which could be advantageous in AFC challenges. The plasma metalloenzyme plasma 5'-nucleotidase indicates small changes in zinc status.[136] Another measure, erythrocyte metallothionein, responds more slowly to low zinc status.

CONCLUSION

Over the past 36 years, research on the effects of AFCs on behavioral and physical markers in children, laboratory animals, and cells has been reported. However, there is no overriding idea about how these mechanisms interrelate. Not only would such an idea help scientists understand the links between AFCs and behavior responses in chil-

dren, it would also shed more light on the basic mechanisms underlying ADHD. Given the known behavioral toxicity of AFCs, it is important to determine why some children may be more sensitive than others to their effects and to identify the tolerable upper limits of exposure for children in general and for children at high risk.

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