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Mini Review

The Safety of Combination of Food Additives in Food Products

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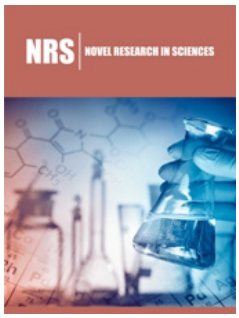

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Abstract

Additives are a group of organic and inorganic compounds that are not raw materials and are used in the production of food in order for the products to be of better quality or longer lasting, to protect the taste, or to improve the taste or appearance. Some of the additives have been used for centuries, for example by salting meat or using CO_2 in wine. The main groups of food additives are antioxidants, colors, flavor enhancers, sweeteners, emulsifiers and stabilizers and preservatives. The FAO has also given a definition of additives, according to which additives are substances that are intentionally added to products, usually in small quantities, have no nutritional value, and the purpose of their addition is to improve the appearance, smell, taste, consistency or durability of the product. Some of the additives that are not approved by the European Commission are approved and used in Australia and New Zealand. Given all of the above, it is easy to conclude that aspects of food safety will be the number one topic in this century. The practical outcome of this review is presented as a set of recommendations for future research in this area. The use of the data in this review is proposed as a training set to develop the framework into a diagnostic tool.

Keywords: Additives; Interaction; Food safety; Combination

Introduction

The use of chemical additives in food is a problem that has been actively considered for a long time. The modern world has established mechanisms for approving additives, but there are still additives for which different parts of the world have different views. The importance of interactions of food additives with other components of food (i.e., nutrients and non-nutrients) has been assessed and certain aspects of toxicology included. With the latest example, the fatal death of a child, from a combination of additives, a new topic is slowly emerging, a new field of work, determining combinations of foods that are beneficial to human health and combinations of foods that are not beneficial to consumer health. A topic that will require an opinion, generally accepted throughout the world.

The Regulation provides for : a) Community 1 lists of approved food additives which are set out in Annex II and III of the Regulation; b) Conditions of use for food additives used in foods, including in food additives, food enzymes as covered by Regulation (EC) No. 1332/2008, food flavorings as covered by Regulation (EC) No.1334/2008 and nutrients; c) rules on the labelling on food additives sold as such; d) specific rules on the "carry-over" principle; e) rules on the la-belling of the so called "Southampton colors". f) specifications (purity criteria) to be established for permitted food additives.

Legal Background of Control of Additives

How are they controlled?

All additives are thoroughly assessed for safety before they are permitted for use, and they are only then permitted to be used in a limited range of products and in certain amounts. These

amounts are based on an Acceptable Daily Intake (ADI) calculated by the European Food Safety Authority (EFSA) from the results of safety tests. The ADI represents an amount that can be ingested daily over a lifetime without appreciable health risk. Approved additives are given a number, and some are also awarded an 'E'. An E shows the additive has been accepted as safe for use within the European Union. Even when an additive has been approved, regular repeat testing is required to maintain its status as 'approved'. Food labels give information about most additives present in the ingredients list, so that consumers can make informed choices.

Some parents report that artificial colors and preservatives trigger hyperactivity in their children, although randomized controlled trials have generally failed to demonstrate a link. However, a study published in 2007 [1] suggested that mixes of certain artificial colors used in foods and drinks together with the preservative sodium benzoate, are associated with hyperactivity in some children, although it is not yet clear whether this is the cause of the hyperactivity. These artificial colors are Sunset yellow (E110), Tartrazine (E102), Carmoisine (E122) Ponceau 4R (E124), Quinoline yellow (E104) and Allura red (E129). Food and drink containing any of these six colors must carry a warning on the packaging. This will say 'May have an adverse effect on activity and attention in children'. The FSA encourage manufacturers to work towards finding alternatives to these colors. Some manufacturers and retailers have already taken action to remove them.

Additives Interaction in Food

The FAO has also given a definition of additives, according to which additives are substances that are intentionally added to products, usually in small quantities, have no nutritional value, and the purpose of their addition is to improve the appearance, smell, taste, consistency or durability of the product. Some of the additives that are not approved by the European Commission are approved and used in Australia and New Zealand. Over the last 30 years in main focus of food safety science is food additive-additive chemical interactions with appropriate relevant information on food additive-food component interactions.

Interactions

One of the major concerns about the safety of dietary supplement ingredients is that interactions between a supplement and other ingested substances (e.g., drugs, other dietary supplements,1 conventional foods) will result in adverse clinical outcomes due to an increase or decrease in the level of the dietary supplement in the organism, an increase or decrease in the level of other xe-nobiotics,2 or combined toxicities. Potential adverse clinical outcomes may result if a dietary supplement lowers a drug's effective concentration. Such a drop in active drug concentration can have serious consequences, especially for persons whose health depends on the therapeutic effects of a drug. Many dietary supplement products are mixtures of two or more substances, some of unknown structure, making an evaluation of interactions more complex, but also more likely to be of clinical concern as they are consumed simultaneously. Interactions can be detected with human, animal, or *in vitro* studies or predicted on the basis of how related substances behave.

Types of interactions

There are numerous mechanisms for interactions among xenobiotics, but most can be categorized as direct chemical-chemical, pharmacodynamic, or pharmacokinetic interactions.

Direct chemical-chemical interactions

The formation of chemical-chemical complexes can modify the action of one or both chemicals. In general, these types of interactions require ingestion of both chemicals within a relatively short time of each other. An example of a direct chemical-chemical interaction occurs in the small intestine, where calcium carbonate taken as a supplement may bind to an acid substance, such as the antibiotic tetracycline, to form an insoluble product. In this case, since the acid was a drug, the action of the drug would be reduced or lost. Other examples include cholestyramine, which adsorbs other drugs, thereby decreasing their availability for absorption, and antacids, which can block iron or zinc uptake. In addition to forming complexes, antacids may significantly change the rate of absorption of other chemicals by altering gastric pH or gastric emptying time, depending on the extent to which pH affects the amount of chemical in the un-ionized state [2].

Interactions with dietary supplements

There are examples of pharmacodynamic interactions that have been noted with dietary supplement ingredients. The antihypertensive effect of guanabenz acetate (a drug used for hypertension) is due to its central agonistic α-2-adrenoceptor activity [3]. Thus, concomitant consumption of yohimbine bark, which contains an α-2-adrenoreceptor antagonist, may diminish the antihypertensive activity of guanabenz through its opposing pharmacodynamic effect. Another example is between the inotropic drug digitalis [4] and Hawthorne leaf or flower; data suggest that both the Hawthorne leaf and the flower may also have a positive inotropic and electrophysiological effect on the heart Schwinger et al. [5]. If digitalis and the hawthorne leaf or flower are taken together, the additive response may be excessive and lead to a serious adverse event [5]. Another additive effect would be exhibited by the ginkgo leaf if its purported antagonism of platelet-activating factor occurred; if ingested with a cyclooxygenase inhibitor, such as aspirin, an in-creased propensity for bleeding would occur [6].

Pharmacokinetic interactions

Pharmacokinetic interactions are interactions that occur when one substance affects the absorption, distribution, metabolism, or excretion of another substance, resulting in altered levels of one of the substances or its metabolites. These interactions include effects caused by the chemicals on xenobiotic metabolizing enzymes and transporters that affect the time course of the concentration of one or both of the chemicals in the body. These interactions commonly take place in the intestines, liver, or kidney and are further categorized based on their site of action.

Altered metabolism

Interactions that alter metabolism warrant attention. Xenobiotics often undergo extensive metabolic alteration by enzymes, resulting in the formation of structurally modified derivatives (metabolites) that may possess different pharmacologic activities (either greater or less) when com-pared with that of the consumed parent compound. There are more than 30 families of xenobiotic metabolizing enzymes in humans, many of which may be limiting for biotransformation of the consumed xenobiotic. If an ingested xenobiotic increases or decreases the amount or activity of a given enzyme, its own rate of metabolism may be altered, as well as that of other consumed compounds. The clinical effect of changes in enzyme metabolism rates will depend on the xenobiotic(s) involved and their metabolites and potencies.

An important group of xenobiotic metabolizing enzymes are the cytochrome P450 (CYP) enzymes, a superfamily of hemoproteins that mediate the biotransformation of 7endogenous and exogenous compounds in the liver, as well as in the intestine and elsewhere. Some CYP isozymes found to be involved with significant pharmacokinetic reactions in humans are CYP1A2, 2C9, 2C19, 2D6, 2E1, and 3A4.In addition, CYP2A6 and CYP2B6 are involved in metabolizing certain xenobiotics (Health Canada, 2000). Since many chemicals are substrates for the same CYP isozymes, one compound may inhibit the activity of the enzyme metabolizing another compound that is ingested concomitantly. In addition, ingestion of a chemical hours before another chemical may induce the production of more enzyme or inhibit normal enzyme synthesis, thus affecting the rate of metabolism of a second chemical metabolized by that same enzyme. While not with-out controversy, grapefruit juice provides one example of an interaction associated with CYP enzymes; it is reported to suppress CYP3A4 and change the concentration of drugs metabolized by the enzyme. When considering dietary supplement ingredient safety, assays for xenobiotic alterations of enzyme metabolism may generate important signals of possible concern, as discussed below.

Altered absorption, distribution, and excretion

Until recently, pharmacokinetic interactions were considered as primarily attributable to the effects on xenobiotic metabolizing enzymes. However, an increasing number of transporters that affect chemical absorption, distribution, and excretion now seem to also play a significant role in pharmacokinetic interactions [7]. Transporters regulate the flux of substances into and out of cells or perform a variety of transmembrane transport functions. Depending on their location and activity, they may have a significant effect on the concentration of a chemical at its site of action.

Interactions between chemicals resulting from competition at transporters are not uncommon. Thus *in vitro* methods to evaluate the effect of chemicals on particular transporters have been developed. Due to differences in human and animal transporters, the methods often employ hu-man transporter proteins expressed in artificial *in vitro* systems, enabling the detailed study of human transporter protein functions with regard to drugs and other xenobiotic substances, including dietary supplement ingredients.

Effects on Excretion

Renal or biliary excretion of xenobiotics, and thus the steady-state plasma concentration of xenobiotics, may also be affected by other xenobiotics. Changes in renal clearance of one xenobiotic can occur through effects of another substance on the urinary pH. Another mechanism for inter-action is the effect of one substance on the active secretion of another substance into the renal tubule. Methods to evaluate the effects of a xenobiotic on excretion are available; they include measurement of tubular uptake, such as perfused kidney assays, or assays at the cellular level. Dietary supplement ingredients that inhibit tubular uptake or in any other way disrupt molecular mechanisms important to excretion of other xenobiotics should be considered of potential concern.

Predicting the potential of ingredients to cause pharmacokinetic interactions

Techniques currently available allow the determination of the extent to which one substance may impact the concentration of other concomitantly ingested substances. There are numerous well-accepted *in vitro* assays designed specifically to determine if a drug may interact with other sub-stances. There are also approaches for describing structures of chemicals likely to cause interactions. These *in vitro* studies and other approaches have focused on determining which drugs affect metabolizing enzymes and transporters and could similarly be used to determine which dietary supplements may lead to interactions. Whether an interaction predicted on the basis of *in vitro* studies actually occurs clinically will depend on whether the dietary supplement compound attains a concentration in vivo adequate to reproduce the effect observed in vitro, as discussed in more detail below.

Invitro prediction of pharmacokinetic effects

In vitro studies for determining which xenobiotics affect transporters and metabolic enzymes ideally employ human transporter proteins or human metabolic enzymes. For example, subcellular fractions of human liver tissue are commonly used, as are whole-cell models such as isolated hu-man hepatocytes, liver slices, and cell lines derived from human cancer cells. Human transporters and enzymes can also effectively be studied by expressing them in other cell types. Changes in either the activity or amount of enzyme or transporter are detected with activity assays, pharmacological assays, and immunochemical or mRNA assays that detect changes in protein or transcription.

In vitro assays for predicting possible interactions are a well-accepted staple of the drug development process. The limitation to using these assays to predict clinical interactions lies, like most *in vitro* assays, in relating the dose at which enzyme or transporter effects are observed with the amount of unbound xenobiotic present at the active site in vivo. If information about the concentration of xenobiotic reached in vivo is available, a comparison of a dietary supplement ingredient's inhibitory binding constants (Ki) for the

CYP enzymes and the in vivo concentration (C_{max}) may place the *in vitro* information in the appropriate perspective.

Animal and human in vivo data in predicting pharmacokinetic effects

Given the inter- and intraspecies differences in xenobiotic metabolizing enzymes, it is ideal to study xenobiotic metabolism using human cells, subcellular fractions of human tissue, or heterologous expressed human proteins, although information about effects on animal proteins may serve as a preliminary indicator of concern. The study of human proteins in transgenic animals may im-prove ability to relate effects observed in animals or animal cells to humans.

Humans themselves may also be studied to determine if a given xenobiotic may cause an observable interaction. Such tests are usually designed to compare the levels of a test substrate with and without the xenobiotic in question. For example, a study of St. John's wort in humans demonstrated that it increased the metabolism of CYP3A4 substrates. Even if specific interaction assays are not done, information about the in vivo concentrations achieved in humans is useful in placing *in vitro* information in perspective.

Databases for Predicting Interactions

Databases helpful for identifying substances likely to interact with other substances have been organized. For example, the database produced by the University of Washington is useful for locating information about potential interactions of particular dietary supplements with other sub-stances. The database also organizes information, such as drug effects on CYP enzymes, that may be useful for identifying potential interactions between particular drugs and supplements. A publicly available website at the Indiana University School of Medicine provides information about drugs metabolized by specific P450 isoforms.

Vulnerable subpopulations

Some individuals are particularly sensitive to adverse effects from xenobiotic interactions be-cause of polymorphic differences that affect the metabolism of some xenobiotics. There are recognized genetic polymorphisms that account for diminished or absent expression of one or more forms of xenobiotic-metabolizing enzymes. There are documented adverse effects directly resulting from the altered metabolism of certain drugs metabolized by these enzymes. A well-known example is people who exhibit little or no CYP2D6 activity in the liver because of inherited genes defective in expression of this form of CYP-a condition that affects 7 to 10 percent of Caucasians, by one estimate. As a result, such individuals are found to experience toxic effects from ordinary doses of the antihypertensive agent debrisoquine, as well as many other drugs for which metabolic elimination is primarily catalyzed by CYP2D6.

It would be reasonable to expect that any dietary supplement ingredient dependent on CYP2D6 for metabolic conversion could potentially produce toxic effects in such persons. Numerous other polymorphisms in xenobiotic metabolism have been or are being identified. Such data can serve to identify people who may be particularly sensitive to dietary supplements cleared by these polymorphic xenobiotic metabolizing systems. Published work on sulfur dioxide and ascorbic and nitrous acid reactions with other food additives to form stable compounds. In some cases, such as between nitrite and sorbic acid, the compounds formed have a potentially higher toxicity than the original additives. No adverse effects have been demonstrated in real foods, however, probably due to the adoption of substantial safe-ty margins between no-effect levels in animals and the maximum levels of additives to which humans could be exposed.

The reactions discussed in this review are those most likely to occur in current additive usage. However, due to the large numbers of permitted food additives, many more interactions occur in foods that could lead to chemical reactions under favorable conditions. Food additives are widely used for technological purposes and their presence is often substantial daily diet. They have also been accused of various toxic reactions in humans. The toxicity of the food color tartrazine, the preservatives sodium nitrate and sodium benzoate, and the antioxidant BHT, was studied using the protozoan Tetrahymena pyriformis as a toxicological model. The 4 food additives were added to Tetrahymena cultures and DNA content of the protozoan nuclei measured by an image analysis system. These food additives caused a statistically significant in-crease in DNA content suggesting stimulation of the mitotic process. This system may contribute to the investigation of the cellular action of food additives, since mitogenic stimuli substantially alter susceptibility to chemical carcinogenesis.

Breast Cancer Resistance Protein (BCRP), multidrug resistance associated protein 2 (MRP2) and P-Glycoprotein (P-GP) are ABC transporters that are expressed in the intestine, where they are involved in the efflux of many drugs from enterocytes back into the intestinal lumen. The inhibition of BCRP, MRP2, and P-GP can result in enhanced absorption and exposure of substrate drugs. Food additives are widely used by the food industry to improve the stability, flavor, and consistency of food products. Although they are considered safe for consumption, their interactions with intestinal transporters are poorly characterized. Therefore, in this study, selected food additives, including preservatives, colorants, and sweeteners, were studied *in vitro* for their inhibitory effects on intestinal ABC transporters. Among the studied compounds, several colorants were able to inhibit BCRP and MRP2, whereas P-GP was fairly insensitive to inhibition. Additionally, one sweetener was identified as a potent inhibitor of BCRP. Dose–response studies revealed that the IC50 values of the inhibitors were lower than the estimated intestinal concentrations after the consumption of beverages containing food colorants. This suggests that there is potential for previously unrecognized transporter-mediated food additive-drug interactions.

Different foods possess different bioactive compounds with varied antioxidant capacities. When foods are consumed together, the total antioxidant capacity of food mixtures may be modified via synergistic, additive, or antagonistic interactions among these components, which may in turn alter their physiological impacts.

Eleven foods from three categories, including fruits (raspberry, blackberry, and apple), vegetables (broccoli, tomato, mushroom, and purple cauliflower), and legumes (soybean, adzuki bean, red kidney bean, and black bean) were combined in pairs. Four assays (total phenolic content, ferric reducing antioxidant power, 2,2-diphenyl-1-picrylhydrazyl, radical scavenging capacity, and oxygen radical absorbance capacity) were used to evaluate the antioxidant capacities of individual foods and their combinations. The results indicated that within the same food category, 13, 68, and 21% of the combinations produced synergistic, additive, and antagonistic interactions, respectively, while the combinations produced 21, 54, and 25% synergistic, additive, and antagonistic effects, respectively, across food categories. Combining specific foods across categories (e.g., fruit and legume) was more likely to result in synergistic antioxidant capacity than combinations within a food group. Combining raspberry and adzuki bean extracts demonstrated synergistic interactions in all four chemical-based assays. Compositional changes did not seem to have occurred in the mixture. Results in this study suggest the importance of strategically selecting foods or diets to maximum synergisms as well as to minimum antagonisms in antioxidant activity.

Exposure to non-nutritional food additives during the critical development window has been implicated in the induction and severity of behavioral disorders such as Attention Deficit Hyperactivity Disorder (ADHD). Although the use of single food additives at their regulated concentrations is believed to be relatively safe in terms of neuronal development, their combined effects remain unclear. We therefore examined the neurotoxic effects of four common food additives in combinations of two (Brilliant Blue and L-glutamic acid, Quinoline Yellow and aspartame) to assess potential interactions. Mouse NB2a neuroblastoma cells were induced to differentiate and grow neurites in the presence of additives. After 24 h, cells were fixed and stained, and neurite length measured by light microscopy with computerized image analysis. Neurotoxicity was measured as an inhibition of neurite outgrowth. Two independent models were used to analyze combination effects: effect additivity and dose additivity. Significant synergy was observed be-tween combinations of Brilliant Blue with L-glutamic acid, and Quinoline Yellow with aspartame, in both models. Involvement of N-methyl-D-aspartate (NMDA) receptors in food additive-induced neurite inhibition was assessed with a NMDA antagonist, CNS-1102. L-glutamic acid- and aspartame-induced neurotoxicity was reduced in the presence of CNS-1102; however, the antagonist did not prevent food color-induced neurotoxicity. Theoretical exposure to additives was calculated based on analysis of content in foodstuff, and estimated percentage absorption from the gut. Inhibition of neurite outgrowth was found at concentrations of additives theoretically achievable in plasma by ingestion of a typical snack and drink. In addition, Trypan Blue dye exclusion was used to evaluate the cellular toxicity of food additives on cell viability of NB2a cells; both combinations had a straightforward additive effect on cytotoxicity. These data have implications for the cellular effects of common chemical entities ingested individually and in combination. The association of food additives with hyperactivity is a popularly accepted notion. Feingold hypothesized that food dyes are pharmacologically active substances that induce or aggravate symptoms of hyperactivity in children. Subsequent studies have confirmed that food colors can induce clinical symptoms of hyperactivity and can also alter brain electrical activity in a subgroup of children with ADHD.

Yet there is still no conclusive scientific evidence to indicate that any of the currently available food additives have any adverse effect on human development. The present study investigated the developmental neurotoxic effects of four common food additives. Two independent models were used to assess interactions in this study: “effect additivity” and “dose additivity.” Combinations acted synergistically in reducing the length of neurite outgrowth from differentiating mouse NB2a neuroblastoma cells. Quinoline Yellow and aspartame showed greater synergy than Brilliant Blue and L-glutamic acid; however, the results indicate that both combinations are potentially more toxic than might be predicted from the sum of their individual compounds.

The colors examined in this study are synthetic dyes that are certified as safe and are permitted for use as food additives in the U.K. Brilliant Blue (E133) is banned in the majority of the EU countries and causes mitochondrial toxicity in vitro. The use of Quinoline Yellow (E104) in foods is banned in Australia, Norway, and the U.S., and genotoxic effects have been reported [8]. However, very little information about the neurotoxicity of food colors is available, and the mechanism by which they exert their toxic effect on nerve cells is not clear.

In contrast, the Excitatory Amino Acids (EAA), L-glutamic acid, and aspartic acid are well established neurotoxins. Over three decades ago, it was discovered that L-glutamic acid destroys dendrites and cell bodies of neurons in the developing brain, thus causing brain lesions. Oral and subcutaneous administration of L-glutamic acid to infant animals (rodents and primates) induces acute neuronal necrosis in several regions of the developing brain including the hypothalamus and the hippocampus. As adults, treated animals show stunted skeletal development, obesity, and female sterility. Retinal neuronal changes also occur in rats after prolonged administration of high L-glutamic acid diets, whilst in adult humans, it elicits headache in susceptible individuals and is believed to be responsible for the “Chinese Restaurant Syndrome” symptoms of which include chest pain, numbness, burning and facial pressure.

Similar hypothalamic lesions can be induced by aspartic acid, one of two of the constituent amino acids in the dipeptide sweetener aspartame. Following ingestion, aspartame is rapidly hydrolyzed to release three biologically active chemicals: aspartic acid, phenylalanine, and methanol, which are absorbed into the portal blood. It has been commonly used in diet drinks and sugar-free foods throughout the world for over 20 years, despite reports of panic attacks, seizures, and headaches with its use. Recently, chronic exposure of aspartame was found to affect memory in rats [9].

Excitotoxins destroy central neurons by excessive stimulation of postsynaptic excitatory mem-brane receptors whereas the under-stimulation of such receptors during the developmental period triggers apoptosis. Thus, excitotoxic and apoptotic neurodegeneration are two distinct cell death processes that are readily distinguishable ultra-structurally. It is well established that an excitotoxic mechanism plays a role in many neurologic disorders, from acute insults such as stroke and head trauma to chronic neurodegenerative states such as Huntington's disease and the acquired immunodeficiency syndrome (AIDS) dementia complex. The over-stimulation of such receptors leads to the opening of voltage-dependent calcium channels, initiating a cascade of events in-volving the activation of protein kinases, phospholipases, proteases, Nitric Oxide Synthase (NOS), generation of free radicals and mitochondrial damage. The NMDA receptor plays a prominent role because of its high permeability to Ca^{2+}; however other EAA receptor subtypes also contribute to these processes. Selective non-competitive NMDA antagonists such as MK-801 markedly protect CNS neurons against direct excitotoxic effects; this has been demonstrated in primary cultures of hippocampal neurons following L-glutamic acid exposure. Our data are consistent with a role for excitotoxicity in the mechanism of injury caused by some flavor-enhancing food additives. CNS-1102 (a NMDA receptor antagonist) protected against both L-glutamic acid and aspartame-induced neurite inhibition, whilst the results demonstrated that food color-induced neurotoxicity was not mediated by NMDA receptor activation. When assessing cell death mechanisms of food additive combinations, we found that both combinations studied had a straightforward additive effect on cell viability, as measured by Trypan Blue dye exclusion. The mechanisms of synergistic neurotoxicity are therefore unrelated to effects on viability.

The list of non-nutritional additives in foods is extensive, and it is virtually impossible to hold a single chemical responsible for a particular dysfunction. For many of the commercial products analyzed, more than one additive was detected. Children's sweets were found to contain both Brilliant Blue and Quinoline Yellow, whilst corn snacks were found to contain both aspartame and L-glutamic acid. Humans are not only exposed to such simple mixtures, but also to complex mixtures of chemicals rather than to individual chemicals, yet they continue to be tested for toxicity in isolation from each other. Also present in the environment are numerous potentially neurotoxic compounds such as pesticides that get into foods somewhere along the chain from farm to plate. It has been estimated that we have in our bodies between 300 and 500 chemicals that did not exist 50 years ago. Thus, mixture studies are important to elucidate whether these interactions or chronic exposure to such mixtures would cause deleterious effects to a developing child. Very few long-term experiments have been attempted, and cumulative toxic effects have hardly been explored at all.

Despite being a major factor relevant to clinical settings, combination pharmacology is a topic that has not received much attention. It is essential that such investigations are carried out by reliable experimental procedures and appropriate statistical methods; however, there is widespread disagreement over terminology, definitions, and models for the analysis of interactions. Several methods for calculating the expected combination effect of two or more compounds are currently in use, the majority of which can be associated with two popular basic concepts known as effect additivity and dose additivity. Effect additivity focuses on measuring the effects of mixtures at only one specified concentration for each compound, thus lacking the information on concentration response relationships. Dose additivity is an equally valid procedure for analyzing interactions between agents irrespective of their mechanisms of action and aims to establish the re-quired concentrations of individual compounds within a combination that produces a specified level of effect. However, this method requires tedious testing with a variety of concentrations for the determination of each data point on the is bologram, where a vast amount of information is eventually lost. Furthermore, is bolographic analysis requiring independent statistical analysis, which can be extremely complicated. There is no generally accepted agreement as to which of the two concepts is more appropriate; therefore, we have attempted to carry out this study using both models to confirm our findings. Similar conclusions could be drawn from both methods.

During the developmental period of synaptogenesis (brain growth spurt period), neurons are very sensitive to specific disturbances in their synaptic environment [10]. In humans, this period ex-tends from the sixth month of gestation to several years after birth, thus children are considerably more vulnerable to harm from toxic chemicals than adults. Since they are at a crucial stage of development, exposure to toxic chemicals may directly or indirectly attack their undeveloped nervous, immune, and endocrine systems. Dysfunction in any of these systems may lead to deleterious health effects. Cell proliferation, migration, differentiation, and synapse formation progress in a tightly programmed and orderly fashion. Interference with any stage of this cascade of events may alter normal progression of subsequent stages and short-term disruptions may have long-term effects later in life. Neurotoxicants may interfere with brain development and subsequent function at exposure levels that have minimal or no effect on the adult brain.

The *in vitro* cell line may of course be more susceptible to toxicity than an in vivo model. Specifically, the *in vitro* neurotoxicity assay has no representation of the blood-brain barrier (BBB); however, this is not complete in the developing human brain until around six months after birth. Furthermore, some regions of the brain are not protected by a BBB at any time in life [10]; thus they remain in contact with any potentially neurotoxic substances circulating in the blood. Such regions are known as the Circumventricular Organs (CVOs), which include the portal system of the hypothalamus. The CVOs make up a minor proportion of the brain but are functionally very important regions.

For the measurement of potential body concentrations following ingestion, a number of assumptions have been made in our calculations. Absorption and distribution of additives need to

be taken into account when relating *in vitro* data to in vivo effects, however there is little information about the absorption from the gut in infants or their distribution in the brain. In all cases, the potential whole body volume exposure to individually assessed additives lies within the range that we found to reduce neurite outgrowth by approximately 10-20% for Quinoline Yellow, 40-50% for Brilliant Blue and aspartame, and 50-60% for L-glutamic acid. Furthermore, neurite outgrowth would be reduced significantly more if the compounds were assessed in combination.

In conclusion, we present evidence that specific combinations of common food additives show synergistic effects to inhibit neuronal cell differentiation in vitro, using both the effect additivity and dose additivity models of assessing interactions. The immature nervous system may be vulnerable to such toxic insults since this marker of neurotoxicity was found at concentrations of additives theoretically achievable in plasma by ingestion of a snack and/or drink typically consumed by children. Mechanisms of synergistic toxicity have yet to be determined, and the implications of these data on developmental disorders remain to be investigated.

Conclusion

Dietary supplements have a potential to adversely affect public health by interacting with other substances. Whether this concern is addressed by labeling precautions, withdrawal of such dietary supplements from the market or requiring warning labels related to usage with other xenobiotics is a regulatory decision. Pharmacists and physicians are made aware of drugs and foods that can potentially interact with other drugs, and drug labeling warns about potential problems. There is no analogous prescribed mechanism to prevent dietary supplement-mediated interactions. A number of pieces of information can suggest a possible interaction between a dietary supplement ingredient and other substances. The potential seriousness of these interactions varies and is placed in perspective by considering if a particular interaction leads to serious adverse events and the likelihood that the interaction will occur.

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