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Methyl aspartylphenylalanine, the pons and cerebellum in mice: An evaluation of motor, morphological, biochemical, immunohistochemical and apoptotic effects

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ABSTRACT

In this study, adult mice were assigned to five groups, and administered vehicle (distilled water), or aspartame (20, 40, 80 and 160 mg/kg body weight) for 28 days. Behavioural tests to assess motor-balance and gait were conducted on day 28, following which animals were sacrificed. Sections of the cerebellar cortex and pons were processed, for general histology and Bielschwosky's silver staining protocol. Glial fibrillary acidic protein (GFAP) and neuron-specific enolase (NSE) immunoreactivity were assessed. Antioxidant status and aspartic acid/cysteine-aspartic acid protease (caspase)-3 levels were also assessed using homogenates of the cerebellum and pons. Body weight-gain decreased significantly following aspartame consumption; while no significant changes in gait and balance were observed. Histological changes suggestive of neuronal injury were observed at 80 and 160 mg/kg/day; however, no obvious neuritic plaques were seen. GFAP-reactive astrocytes and NSE-reactive neurons increased at 20, 40 and 80 mg/kg, but decreased at 160 mg/kg. There was derangement of oxidative status and increased caspase-3 concentration with increasing doses of aspartame; although no significant difference in aspartate level was observed. The study concluded that repeated oral administration of the higher doses of aspartame was associated with morphological alterations suggestive of neuronal injury, and derangement of antioxidant status.

1. Introduction

Non-nutritive sweeteners (NNS) are highly-potent sugar substitutes that ensure palatability of foods and beverages while maintaining a low caloric value. In recent times, addition of sugar to foods or beverages has been associated with some negative health outcomes (Malik et al., 2010; Te Morenga et al., 2013), thereby increasing the clamour for substitution of sugar with NNS (Mattes and Popkin, 2009; Tate et al., 2012), whenever possible. However, there are ongoing controversies over the possible health consequences of consumption of NNS (Tandel, 2011). These are reflected in reports of potential health benefits (Mattes and Popkin, 2009; Fernstrom, 2015), deleterious effects (Fowler et al., 2008; Swithers, 2013), and effects that are yet unclear (Renwick and Molinary, 2010; Pepino and Bourne, 2011). A few animal studies have reported body weight gain (instead of weight loss), after consumption of NNS (Swithers and Davidson, 2008); while in others, NNS have been associated with cancers (Zwillich, 2007).There are also studies that have reported possible deleterious effects of some NNS on the brain. Cong et al. (2013) reported that chronic (40 weeks) oral consumption of acesulfame potassium was associated with neurosynaptic and/or metabolism-related genomic and proteomic alterations in the hippocampus, and impaired hippocampal-dependent learning. Christian et al. (2004), Bergstrom et al. (2007) and Omar (2009) have also demonstrated that consumption of aspartame was associated with behavioural, biochemical and morphological alterations in the brain.

Aspartame is one of the six NNS (aspartame, sucralose, saccharin, acesulfame potassium, neotame and advantame) that have been approved for use as sweeteners in food. Aspartame is an intensely-sweet, odourless, white, crystalline powder, which is an important component of a number of foods and beverages (Magnuson et al., 2007). It is one of the most widely-used NNS (Abdel-Salam et al., 2012). Aspartame was approved for use in the United States of America in 1981 (Stegink, 1987), and in Europe in 1994 (Butchko et al., 2002); and it is reportedly one of the most exhaustively-studied substances in the human food supply (FDA, 2015). However, despite continued evaluation and re-evaluation (FDA, 1984, 2006, 2015; EFSA, 2006) of the health

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implications of aspartame consumption, its safety remains the subject of several debates; with researchers in support of (Molinary, 1984; Kotsonis and Hjelle, 1996; Butchko et al., 2002) and against it (Soffritti et al., 2006; Simintzi et al., 2007; Humphries et al., 2008).

The safety of aspartame and its metabolic breakdown products (phenylalanine, aspartic acid and methanol) has been assessed repeatedly in humans; and presently, international regulatory agencies and expert committees agree that from the weight of available scientific evidence, it is clear that aspartame is safe for its intended use (Butchko et al., 2002); however, this seems not enough to quieten "anti-aspartame" critics. In rodents, a number of acute and chronic studies have reported that aspartame is not carcinogenic or genotoxic at doses within or which far exceed the acceptable daily intake (Ishii, 1981; Durnev et al., 1995; Mukhopadhyay et al., 2000). Effects of aspartame on the rodent brain have also been studied extensively, although these studies have failed to reach a consensus. There have been reports suggesting that aspartame does not have deleterious effects on the brain or brain neurotransmitter levels (Finkelstein et al., 1988; Dailey et al., 1991), while some other studies have shown morphologic or biochemical evidence of neuronal injury (Goerss et al., 2000; Beck et al., 2002; Omar, 2009; Abd El-Samad, 2010; Abu-Taweel et al., 2014). In two recently-published studies from our laboratory, we reported dose-related behavioural, biochemical and morphological changes involving the cerebral cortex (Onaolapo et al., 2016a) and hippocampus (Onaolapo et al., 2017a); following repeated administration of increasing doses of aspartame in mice. At the lower doses (20 and 40 mg/kg), aspartame did not significantly alter morphology, morphometry or biochemistry of neurones in the hippocampus or cerebral cortex; and curiously, it was associated with improvement in spatial working-memory in the radial arm maze and Y-maze. However, at higher doses (80 and 160 mg/kg), there were changes suggestive of neuronal injury in the cerebrum and hippocampus.

The results of these studies piqued our interest in examining the possible effects of aspartame on other brain regions {such as the cerebellum and/or brain stem (pons)} when aspartame is administered repeatedly by gavage. Abd El-Samad (2010) reported microscopic evidence of cerebellar injury following oral administration of aspartame at 250 mg/kg (a dose significantly higher than what was used in this study) in rats. However, there is a dearth of scientific literature on the possible effects of aspartame on the cerebellum or pons at the doses examined in the present study.

Studies assessing aspartame consumption across all age groups concluded that daily intake of aspartame (even at the 90th percentile) was only about 5–10% of the ADI in the United States (ADI 50 mg/kg), and less than this in a number of European countries (ADI 40 mg/kg), and Brazil (Bar and Biermann, 1992; Toledo and Ioshi, 1995; Garnier-Sagne et al., 2001; Butchko and Stargel, 2001). However, there are strong indications that with the increase in the number of aspartame-containing products, there is an increased chance of individuals inadvertently consuming such products (Humphries et al., 2008), or simultaneously consuming several aspartame-containing food products which could increase the risk of reaching or exceeding the acceptable daily allowance. Thus, there is a need for continued research into the potential health implications or benefits of aspartame consumption; either from a medical point of view, or for the purpose of improving our understanding of aspartame.

Therefore, the rationale for this study was the need to ascertain the effects of repeated daily administration of vehicle (distilled water) or aspartame at 20–160 mg/kg body weight/day (Onaolapo et al., 2016a, 2017a) on body weight, motor-coordination, neurochemistry, antioxidant status, caspase-3 concentration, and morphology/morphometry of the cerebellum and pons in mice that were maintained on standard pelletised chow and tap water (*ad libitum*). We tested the hypothesis that repeated oral administration of increasing doses of aspartame could significantly alter motor-coordination, brain aspartic acid and caspase-3 levels, antioxidant activity and morphology/morphometry of the cerebellar cortex and pons in mice.

2. Methods

2.1. Chemicals

Aspartame tabletop sweetener (99.9% purity, NutraSweet®, Nutra Sweet Company, Illinois, USA). Caspase-3 colorimetric assay kit (Yeasen, China), aspartic, acid, superoxide dismutase, glutathione, nitric oxide and malondialdehyde assay kits (BioVision Incorporated, CA, USA).

2.2. Animals

Male Swiss mice (Empire Breeders, Osogbo, Osun State, Nigeria), weighing 25–30 g each were used for this study. Mice were housed in groups of 6 in plastic cages located in a temperature-controlled room (22–25° Celsius) with 12 h of light daily (lights on at 7 a.m). The animals were fed commercial (Top Feeds®, Premier feeds Ltd, Nigeria) standard chow (Calories: 29% protein, 13% fat, 58% carbohydrate) from weaning and had access to tap water *ad libitum*. Mice received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health National Institutes of Health (2013). All procedures were conducted in accordance with the approved institutional protocols and within the provisions for animal care and use prescribed in the scientific procedures on living animals, European Council Directive (EU2010/63).

2.3. Experimental method

60 adult male mice were assigned into 5 groups of 12 animals each. Mice were administered vehicle (distilled water) or one of four doses of aspartame (20, 40, 80 and 160 mg/kg body weight) daily, for 28 days (Onaolapo et al., 2016a, 2017a). Doses of aspartame to be administered to each mouse was calculated, measured, and then dissolved in distilled water. Vehicle or aspartame was administered by gavage. Mice were weighed weekly (7:00 am before feed hoppers were refreshed with food) using a weighing balance (Mettler Toledo Type BD6000, Greifensee, Switzerland). At the end of the experimental period, behavioural tests to assess motor-balance and gait were conducted, after which animals were sacrificed by cervical dislocation, following anaesthesia with isoflurane. Brains were dissected out, observed grossly, blotted dry and either fixed in 10% neutral buffered formalin for histology, or homogenised for assessment of aspartic acid/caspase-3 levels, and brain antioxidant status.

2.4. Behavioural tests

Behavioural tests were conducted on day 28 (after last dose of aspartame or vehicle); thirty minutes (Onaolapo et al., 2016a, 2017a) after administration of vehicle or aspartame. Tests were conducted in a quiet room between the hours of 8 a.m. and 2 p.m. On each of the test days, mice were transported in their home-cages to the behavioural test laboratory, allowed to acclimatise for 30 min, and then administered aspartame or vehicle. The behavioural parameters were later scored by two independent observers who were blind to the groupings.

2.4.1. Beam-walking test

Performance on the balance-beam is a validated measure of fine coordination and balance. The beam test has been used for the detection of deficits that accompany aging, pharmacological manipulations and lesions of the central nervous system (Luong et al., 2011). Mice with cortical impact lesions may slip repeatedly on the beam (Buccafusco, 2009). Motor-coordination and balance were assessed as described by Southwell et al. (2009) and Luong et al. (2011), based on