ALTERATIONS IN BRAIN HISTOMORPHOLOGY AND SOME HOMOGENATE ANTIOXIDANT BIO-POINTERS IN L-ARGININE CO-EXPOSED ASPARTAME-ASSAULTED RATS

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Received *May 14, 2021;* ***Revised*** *July 15, 2021;* ***Accepted*** *July 19, 2021*

ABSTRACT

Assault-related oxidant effect of aspartame, an excitatory-non-nutritive sweetener, could be influenced by L-arginine, the sole precursor of a conditional antioxidant, nitric oxide. Herein, alterations in brain histomorphology and some homogenate antioxidant bio-pointers in L-arginine co-exposed aspartame-assaulted rats' were evaluated in thirty male Wistar rats by standard protocols. Group A rats, control, were exposed to distilled water and had free access to feed. Groups B, C, D, E and F rats were, respectively exposed to aspartame (1000 mg/kg), aspartame (1000 mg/kg) plus Vitamin C (100 mg/kg), L-arginine (20 mg/kg), aspartame (1000 mg/kg) plus L-arginine (20 mg/kg) and aspartame (1000 mg/kg) plus L-arginine (40 mg/kg). Exposure to aspartame for twenty-one days caused a significantly increased ($p<0.05$) catalase and superoxide dismutase activities, reduced glutathione, thiobarbituric acid reactive substance and total protein concentrations but non-significantly reduced ($p>0.05$) ferric reducing antioxidant power in the rats' brain homogenate, compared to others. Brain histology of Groups A and F rats were preserved, compared to others. Thus, aspartame significantly compromised the determined antioxidant bio-pointers and histology while L-arginine particularly at 40 mg/kg ameliorated same in the rats' brain via apparent oxidant and antioxidant mechanism respectively.

Keywords: Aspartame, L-Arginine, Vitamin C, Toxicity, Brain histomophology, Rats

INTRODUCTION

The use of artificial sweeteners in place of sucrose is on the increase (Lohner *et al*., 2017) as artificial sweeteners are not strongly associated with unsafe health issues (Toews *et al*., 2019). Generally, sweeteners ensure reduced sugar, hence caloric intake without altering the palatability of foods or drinks. However, as none provides full satisfaction, consumers may be predisposed to unintentional abuse with consequent health issues (Chattopadhyay *et al*., 2014). In particular, unintentional abuse of aspartame has been reported (Humphries *et al*., 2008).

Generally, aspartame is an odorless, highly-sweet, white, crystalline powdery methyl ester comprising natural amino acids - L-aspartate and L-phenylalanine. It could be hydrolyzed into its constituent amino acids - aspartate and phenylalanine under condition of elevated temperature or pH (Chattopadhyay *et al*., 2014). However, hydrolysis of aspartame by digestive esterases and peptidases in the intestinal lumen yields aspartate, phenylyalanine and methanol (Yagasaki and Hashimoto, 2008). Metabolic fates of these metabolites of aspartame could result to possible toxic influence of aspartame in animals. This is worrisome given that aspartame could cross the

blood brain barrier to attack the brain cells and create a toxic cellular overstimulation. The worry is justified as inferable from the biochemistry of aspartame metabolites. Aspartate is an excitatory amino acid while phenylalanine is the precursor for some catecholamine neurotransmitters, whereas methanol is an alcohol (Yagasaki and Hashimoto, 2008). As an alcohol, methanol oxidation results to the formation of formaldehyde and subsequently fomic acid and formate which are injurious to the cells (Ashurst and Nappe, 2020). Excess of aspartate, as excitatory amino acids, in concert with its inter-conversion products - asparagine, glutamine and notably glutamate, results to hyper-excitability of neurons and eventual death already reported as aspartame-related neurotoxic effects (Soffritti *et al*., 2005; Omar, 2009). In particular, aspartame-derivative, methanol has been associated with depletion of reduced glutathione (GSH) (Ashok and Sheeladevi, 2014). Glutathione depletion in brain is a common characteristic of chronic aspartame administration (Prokić *et al*., 2014).

Generally, overproduction of free radicals accounts for tissue injury, and induction of free radical production is an underlying mechanism of agent-related toxicity. Free radicals are unstable atoms with unpaired valence electrons hence could easily attack biomolecules in the body transforming them into other free radicals including hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) (and even nitric oxide (NO) which is a conditional antioxidant and prooxidant) (Phaniendra *et al*., 2015). It has been reported that a significant increase in the metabolites of aspartame following its consumption accounted for a spike in generation of free radicals and pro-oxidative actions-related neurotoxic effect in rats' brains (Ashok and Sheeladevi, 2014; Prokić *et al*., 2014). Vital body organs, including the brain, are very susceptible to reactive oxygen species, (ROS) generated during cellular oxidative reactions, because of their high metabolic rate, important biochemical functions and high content of oxidizable substrates. In nature, such high metabolic organs are protected from oxidative stress-prone free radicals attack by a well-developed multiple antioxidant defense systems (Poljšak and Fink, 2014). Antioxidants, which could be enzymatic or non-enzymatic and in many foods, including fruits and vegetables, by transferring electron to oxidizing agents, inhibit free radical production and consequent oxidative stress-related cell damage (Valko *et al*., 2006). Oxidative stress, a state of imbalance between the antioxidant and prooxidant in favour of the prooxidant leading to excess free radical generation (ROS) in the body, is fundamental to many diseases (Egbuonu and Ejike, 2017; Soffritti *et al.,* 2007). Generally, oxidative stress includes many central nervous system (CNS) diseases, such as Alzheimer's disease, cerebral ischaemia, traumatic brain injury (TBI), and Parkinson's disease that are traceable to brain dysfunction.

L-arginine is a semi-essential, basic amino acid and a sole substrate for the synthesis of nitric oxide - a neurotransmitter that supports several brain functions (El Mesallamy *et al.,* 2008) apart from participating in anabolic hormone simulation and mediating L-arginine-related protective roles against chronic disease (Lass *et al*., 2002). L-arginine is in common natural foods, including nuts, and synthetic supplements that is commonly used in diets and drugs owing to its possible benefits in animals (Egbuonu and Ejike, 2017). The brain plays central role in endocrine expression and neuronal transmission involved in oxidative stress responses (Salim, 2017). Thus, the indirect conditional antioxidant and pro-oxidant amino acid, L-arginine may be co-consumed with aspartame resulting to unknown consequent effects on the brain histology and antioxidant defense system. These warranted this study aimed at assessing the alterations in brain histomorphology and some homogenate antioxidant bio-pointers in L-arginine co-exposed aspartame-assaulted rats.

MATERIALS AND METHODS

Drugs: Vitamin C (100 mg) tablet was procured from Emzor Pharmaceuticals, Lagos, Nigeria, while L-arginine and aspartame, were products of Sigma Chemical Company, St. Louis, USA.

Animal: Male Wistar rats (totaling thirty six; weight range 50 – 70 g) obtained from the Department of Veterinary Medicine, University of Nigeria, Nsukka were allowed to acclimatize for a period of two weeks in the Department of Biochemistry, Michael Okpara University of Agriculture Umudike, Animal House. The rats were kept at normal environmental temperature and natural day light/darkness cycle. The study was carried out in accordance with the ethical guidelines of the National Research Council, USA (NRC, 2011). The study used completely randomized blocked design of six treatment groups replicated thrice with each replicate having two rats. Rats in group A, control, were exposed to distilled water (vehicle) and had free access to feed (Vital Feed, Growers Marsh having 20 % crude protein and 280 kcal/100g metabolizable energy, Vital Feed Industries Limited, Nigeria) and portable water. Rats in group B, C, D, E and F were, respectively exposed to aspartame (1000 mg/kg), aspartame (1000 mg/kg) plus Vitamin C (100 mg/kg), L-arginine (20 mg/kg), aspartame (1000 mg/kg) plus L-arginine (20 mg/kg) and aspartame (1000 mg/kg) plus L-arginine (40 mg/kg). Exposure was oral through daily oral intubation for twenty one (21) days. The doses were calculated for human and modified for rat using the method of Paget and Barnes (1964). L-arginine solution was prepared by dissolving 200 mg of L-arginine in 10 ml of distilled water to give 20 mg/ml. It was administered at doses of 20 mg/kg and 40 mg/kg respectively. Aspartame solution was prepared by dissolving 10,000 mg of aspartame in 10 ml of water to give 1,000 mg/ml. It was administered at the dose of 1000 mg/kg. Vitamin C solution was prepared by dissolving 1000 mg of ground Vitamin C in 10 ml of water to give 100 mg/ml Vitamin C was administered at a dose of 100 mg/kg.

The Vitamin C co-exposed group was included as a standard because Vitamin C is a dietary water soluble antioxidant that is vital in neurotransmission (Traber and Stevens, 2011). Twenty four hours after the last treatment, the rats were respectively sacrificed after mild anesthesia and the brain sample of the respective rats was excised. Three of the rats' brain per group was homogenated while the other three was fixed for biochemical and histological evaluation, respectively. 10 % brain tissue homogenate was obtained by separately grinding a 0.5 g of each respective brain sample in 5 ml of phosphate buffer saline (pH 7.2), using mortar and pestle. The homogenate was centrifuged at 1000 g for 10 minutes and the supernatant was stored at minus 20 ^{o}C until used for the determination of the studied parameters.

Determination of Some Antioxidants in the Brain Homogenate of Rats: Superoxide dismutase (SOD) activity was assayed by the method of Madesh and Balasubramanian (1998). Catalase (CAT) activity was assayed by the method described by Johansson and Borg (1998). Thiobarbituric acid reactive substance (TBARS) concentration in the brain homogenate of rats was determined using the method of Wallin *et al.* (1993). Ferric reducing antioxidant power (FRAP) in the brain homogenate of rats was determined as described by Benzie and Strain (1999). Reduced glutathione (GSH) concentration in the brain homogenate of rats was determined by the method of Goldberg and Spooner (1983) using Randox kit. Total protein concentration in the brain homogenate of rats was determined with Randox kit based on the Biuret method of Weichselbaum (1946).

Rats' Brain Tissue Preparation for Histopathological Examination: Organ specimen (brain) promptly excised from the humanely sacrificed rats for histological examination were fixed in 10% buffered formal-saline until used. The histological preparation of the brain tissue followed the method described by Egbuonu and Ejike (2017). Photomicrographs were taken using a Motic 9.0 megapixels microscope camera at x400 magnifications.

Statistical Analysis: The data were subjected to one way analysis of variance followed by post-hoc multiple comparison (Dunnett's test). The difference in mean was accepted as statistically significant at $p<0.05$. The results were presented as mean ± standard error of mean (SEM).

RESULTS

The exposure of rats to aspartame for twenty-one days caused a significant increase ($p<0.05$) in TBARS (2.38 ± 0.25 mg/ml) and total protein (0.60 ±05 g/ml) concentration, but did not significantly reduce ($p>0.05$) ferric reducing antioxidant power (0.08 ± 13 µg/mL) in the rats' brain homogenate, compared to others. The least FRAP (0.03 ±01 µg/mL) was recorded in rats exposed to aspartame plus high L-arginine (Table 1).

From Table 2 and as compared to others, the exposure of rats to aspartame for twenty one days caused a significant increase ($p<0.05$) in GSH (10.60 ± 2.97 mg/dl), CAT (14.69 ± 1.86 IU/L) and SOD (0.65 ± 0.04 IU/L) activities in the rats' brain. However, the GSH value obtained in aspartame plus high L-arginine-fed (3.80 ± 1.15 mg/dl) rats was similar to that in the control. The observed CAT value was comparatively lower than that in aspartame plus Vitamin C (15.71 ± 1.42 IU/L) and in aspartame plus low L-arginine (16.98 ± 11.83 IU/L) fed groups and least in the aspartame plus high L-arginine-fed rats (11.75 ± 1.47 IU/L). Also, the SOD value in aspartame plus high L-arginine-fed (0.48 ± 0.03 IU/L) as compared to the control was least compared to that in the other groups.

Exposure to aspartame for twenty-one days revealed that there was no histological lesion in the control, but varying degree of lesion in groups B, C, D and E showing congested central vein. However, that in group D rats was less pronounced compared to that in group B, C and E rats (Figure 1).

DISCUSSION

Aspartame could cross the blood brain barrier to create a toxic cellular overstimulation through its metabolites, including aspartate and methanol, and trigger excessive amounts of free radicals which are cytotoxic with consequent brain cell damage (Jeganathan and Namasivayam, 1998). Thus in this study, the apparently marked elevation in free radicals, indicated by increased activity of SOD and CAT enzymes as well as GSH, total protein and TBARS concentration but decreased FRAP, as observed in aspartame-assaulted rats' brain was not surprising. The observations could be indicating collapsed antioxidant status and function in the aspartame-intoxicated rats following possibly spiked aspartame-intoxication-related aspartate and methanol anabolism and consequent intoxication (Skrzydlewska, 2003; Prabhakar *et al.*, 2005).The increased SOD and CAT activity in aspartame-assaulted rats was in line with the earlier report by Ashok and Sheeladevi (2014).

In particular, the increased CAT activity resulted in enhanced conversion of hydrogen peroxide to hydroxyl radical – a co-contributor to oxidative stress (Reiter, 2000), while that of GSH resulted from reduction of oxidized glutathione (GSSG) by glutathione reductase (Cazenave *et al.*, 2006) most likely as a consequent cellular antioxidant protection activity (against oxidative stress) in the rats' brain. Also, increased cellular GSH following assault-related leakages to the parent tissue may have further increased the brain cell vulnerability to oxidative stress (Oyama *et al.*, 2002). The increased GSH concentration in the aspartame-intoxicated rats' brain homogenate as observed in this study may be a consequence of aspartame-assault-related spike in the metabolites. Such spike in the metabolites could in addition enhance the generation of free radicals that readily react with the amino acids of soluble proteins. It could also lead to the formation of hydroxymethyl and peroxides derivatives of proteins (Skrzydlewska *et al.*, 2003). This could explain the attendant increased protein concentration as observed in the aspartame-assaulted rats with possible diminution in functional capacity.Proteins are vital components of living cells with diverse functions, including transport, immune defense and blood clothing (Amin *et al.*, 2010). The increased protein concentration in aspartame-assaulted rats as observed in this study was in line with the work of Gul *et al.* (2017) that reported significant increase in total protein concentration following aspartame administration in rats.

Table 1: Effect of L-arginine on ferric reducing antioxidant power (FRAP), total protein (TP) and thiobarbituric acid reactive substance (TBARS) concentration in aspartame-assaulted rats' brain homogenate

Group/Treatment	FRAP (µg/mL)	TP (g/ml)	TBARS (mg/ml)
A (Control, distilled water 1 mL/kg)	0.11 ± 0.10^{e}	0.18 ± 0.20^{c}	0.69 ± 0.06^{c}
B (Aspartame 1000 mg/kg)	0.08 ± 1.30^{d}	0.60 ± 0.50^{f}	2.38 ± 0.25^{f}
C (Aspartame 1000 mg/kg + Vitamin C 100 mg/kg)	0.06 ± 0.30^{b}	0.12 ± 0.40^{a}	0.87 ± 0.28^{d}
D (L-arginine 20 mg/kg)	0.07 ± 0.90^{C}	0.23 ± 0.30^{d}	1.42 ± 0.07^{e}
E (Aspartame 1000 mg/kg +L-arginine 20 mg/kg)	0.14 ± 0.30^{f}	0.56 ± 0.30^{e}	0.59 ± 0.02^{b}
F (Aspartame 1000 mg/kg + L-arginine 40 mg/kg)	0.03 ± 0.10^{a}	0.15 ± 0.30^{b}	0.14 ± 0.04^{a}

Means on a column with different superscript letters are significantly different at p<0.05

Table 2: Effect of L-arginine on reduced glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) concentrations in aspartame-assaulted rats' brain homogenate

Group/Treatment	GSH (mg/dl)	CAT (IU/L)	SOD (IU/L)
A (Control, distilled water 1 mL/kg)	3.80 ± 1.30^{c}	12.40 ± 1.06^{c}	0.19 ± 0.04^{a}
B (Aspartame 1000 mg/kg)	10.60 ± 2.97^{f}	14.69 ± 1.86^{d}	0.65 ± 0.04^{f}
C (Aspartame 1000 mg/kg + Vitamin C 100 mg/kg)	3.75 ± 1.71^{a}	15.71 ± 1.42^{e}	0.59 ± 0.10^{e}
D (L-arginine 20 mg/kg)	6.80 ± 2.30^{d}	4.66 ± 1.82^{a}	0.50 ± 0.18^{c}
E (Aspartame 1000 mg/kg +L-arginine 20 mg/kg)	9.20 ± 3.30^{e}	16.98 ± 11.83^{f}	0.52 ± 0.11^{d}
F (Aspartame 1000 mg/kg + L-arginine 40 mg/kg)	3.80 ± 1.15^{b}	11.75 ± 1.47^{b}	0.48 ± 0.03^{b}

Means on a column with different superscript letters are significantly different at p<0.05

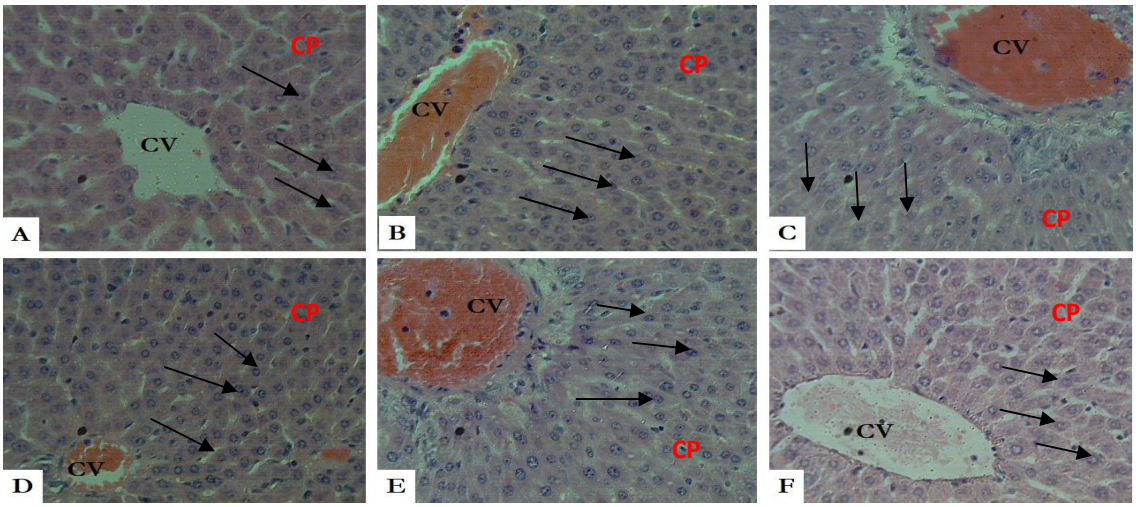

Figure 1: Photomicrograph of effect of L-arginine on aspartame- assaulted rats' brain (cerebellum) (H & E, x400). *CV = Central vein; CP = Cytoplasm space; Arrow heads = Astrocytes (A = Group A; B = Group B; C = Group C; D = Group D; E = Group E and F = Group F rats)*

The least FRAP recorded in rats exposed to aspartame plus high L-arginine suggested apparent overriding scavenging potential in the rats' brain following aspartame plus low L-arginine exposure to the rats (Egbuonu and Ejike, 2017). The aspartame-assault-related increase in TBARS in the rats' brain, compared to control and other groups tallied with earlier report that cellular lipids damaged by ROS, lead to lipid peroxidation and increased generation of TBARS (Yadav and Ramana, 2013). The observed aspartame-related significant increase ($p<0.05$) in SOD and CAT activities in the rats' brain, compared to control and other groups may be a consequence of increased need for concerted conversion of superoxide radicals (O_2^-) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2) by SOD and subsequent conversion of hydrogen peroxide to oxygen and water by CAT, to provide cellular defense against reactive oxygen species. Conversion of superoxide and hydrogen peroxide to water and

molecular oxygen were antioxidant response to oxidative stress in animals (Egbuonu *et al.*, 2017).

There was no histological lesion in the control but varying degree of lesion in groups B, C, D and E rats showing congested central vein. However, that in group D rats was less pronounced compared to that in groups B, C and E. This indicated that L-arginine at 40 mg/Kg may mitigate aspartame-induced adverse effect on the rats' brain histology in apparent confirmation of the homogenate chemistry results of the studied antioxidant bioindicators. Confirmation of clinical chemical results from assessed organ histology had been suggested (Egbuonu and Ejike, 2017).

It is important to note that increased SOD activity does not translate to efficient diminution of oxidative damage (Ashok and Sheeladevi, 2014). Thus, the concurrently increased SOD activity along with other indicators of oxidative stress, particularly, TBARS concentration in this study may be indicating increased production of free radicals in the aspartame-assaulted rats. Enhanced SOD enzymic activity while acting as first line of antioxidant defense against superoxide radicals enhances the conversion of superoxide radicals to another free radical, hydrogen peroxide (H_2O_2) that warrants concerted second line antioxidant defense actions (Ashok and Sheeladevi, 2014). Also, Vitamin C as an enzyme cofactor (Chatterjee, 2009) functions in metabolic activities involved in antioxidant defense, including reduction of oxygen species following lipid peroxidation and protein oxidation as well as preventing nitrosamine formation to reduce DNA damage (Traber and Stevens, 2011). Results from this study, however, demonstrated that L-arginine at highest tested dose out-compared Vitamin C in mitigating aspartame-assault-related effect on the rats' brain histology and tested antioxidant parameters. These results suggested significant L-arginine-mediated antioxidant effect and capacity to reverse aspartame-assault-related oxidant observations. However, the preserved brain histology in the control and aspartame plus L-arginine (40 mg/kg) co-exposed rats, compared to others indicated selective ameliorative capacity of L-arginine on aspartame-assault-related rats' brain histology damage.

Conclusion: Thus, aspartame significantly compromised the determined antioxidant bio-pointers and histology while L-arginine particularly at 40 mg/kg ameliorated same in the rats' brain *via* apparent oxidant and antioxidant mechanism, respectively. These, while advocating cautious use of aspartame, warrant further studies to harness highlighted benefits in L-arginine co-exposed aspartame-assaulted rats.

ACKNOWLEDGEMENTS

The authors sincerely acknowledge the academic staff, laboratory technologists and histopathologist in the relevant departments and colleges of Michael Okpara University of Agriculture Umudike for the important input and guidance while this study lasted.

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