Comparative Study on The Safety of Aspartame and Stevia on The Adrenal-Pituitary Axis of Adult Male Albino Rats: Histological and Immunohistochemical Study

Nesreen G. Abdelhalim and Doha S. Mohamed

Histology Department, Faculty of Medicine, Sohag University, Egypt.

ABSTRACT

Introduction: Aspartame is the most widely used artificial sweeteners. Stevia is a worldwide natural sweetener plant with medicinal and commercial importance. Stress responses are mediated both in the central and peripheral nervous system. The principal effectors of the stress response are localized in the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal gland. This is commonly referred to as the hypothalamic-pituitary-adrenal axis.

Aim: To compare the effect of aspartame and stivea on the pituitary adrenal axis.

Material and Methods: A total of 30 adult male albino rats were equally divided into three groups. Group I was the Control Group. Group II (ASP Group) received aspartame (ASP), at daily oral dose 250 mg/kg for 4 weeks. Group III (stevia Group) received stevia at daily oral dose 250 mg/kg for 4 weeks.

Results: In aspartame-treated group there was apoptosis of zona fasciculata cells with subsequent corticotrophic hyperplasia. In stevia treated groups minimal changes were observed with no significant changes in number of corticotrophes.

Conclusion: As a natural sweetener, stevia is safer than aspartame on the adrenal pituitary axis.

Keywords: Adrenal, aspartame, stivea, Zona fasciculata

Revised: 31 May 2016, Accepted: 06 July 2017

Corresponding Author: Doha Saber Mohammed, Department of Histology, Faculty of Medicine, Sohag University, Sohag, Egypt., Tel.01007843762, E-mail: dohasaber@yahoo.com

ISSN: 1110-0559, 2017, Vol 40, No. 2

INTRODUCTION

Aspartame (ASP) is the most widely used artificial sweetener. It is a methyl ester of a dipeptide (L-aspartyl-L-phenylalanine methyl ester). It is a component in many foods. It had about 200 folds higher sweetness than sucrose[1]. ASP is hydrolyzed in the intestine to its components; phenylalanine, aspartate and methanol. Each one of these components is toxic and affects different body organs[2].

Stevia is a natural sweetener plant having medicinal and commercial importance. It is frequently used all over the world[3]. Stevia is formed of sweet diterpene glycosides: rebaudioside A, rebaudioside C, stevioside and dulcoside in its leaf tissue[3].

Stress response is mediated by sweeteners in the central nervous system and peripheral tissues, both. The principal effectors of the stress response are localized in the para-ventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland, and the adrenal gland. This collection of structures is commonly referred to as the hypothalamic-pituitary-adrenal (HPA) axis[4, 5].

AIM OF THE WORK

This work aimed to compare the effect of aspartame and stivea on the pituitary adrenal axis.

MATERIAL AND METHODS

Animals used:

A total of 30 adult male albino rats (200250-g) brought from the animal house of the Faculty of Medicine, Assiut. They were reared under the standard conditions of feeding, light-dark ratio and temperature, at Faculty of Medicine animal house, Sohag, Egypt.

The animals were divided into three equal groups, ten rats each:

Group I (Control Group) received 250 ml D.W for 4 weeks

Group II (ASP Group) received ASP, at daily dose 250 mg/kg dissolved in distilled water and given orally to the animals by intra-gastric tube for 4 weeks (6).

ASP tablets, each one containing 20 mg, were obtained from Al-Ameriya Pharma Company. This dose
was calculated according to the human dose, which is 4050-50 mg/kg per day, after species factor correction [7].

**Group III (stevia Group)** received stevia obtained from pyure company, at a daily oral-dose of 250mg/kg by intra gastric tube for 4 weeks.

The extract was prepared by boiling 25g of stevia leaf powder in 500ml D.W for one and a half hour till reduced to 150 ml (8).

**Methods:**

At the end of the experiment, the rats were anesthetized using ether inhalation, sacrificed, carefully dissected, and adrenal ad pituitary gland were taken for light, immunohistochemical and electron microscopic examination.

**Preparation of the specimens for light microscopic examination (9):**

Perfusion fixation is used and the specimens was fixed in 10% neutral buffered formalin and processed for light microscopic study to get paraffin sections of 6μm thickness for general and immunohistochemical stains

**Immunohistochemical methods:**

Immunohistochemical staining was carried by avidin biotin peroxidase complex method. Specimens from pituitaries and adrenals were processed. Adrenocorticotropic hormone (ACTH) Ab-1 and caspase-3 (purcashed as anti-ACTH, mouse monoclonal antibody and anti-cleaved caspase-3 rabbit polyclonal antibody, Neomarkers Fermont, CA 94539, USA) were used for detection of corticotrophs and apoptotic cells respectively. The reaction appeared brownish either cytoplasmic or nuclear.

Sections were then counterstained with Mayer’s hematoxylin, dehydrated, cleared, and mounted. Tonsils were used as the positive-control tissues. Negative control lacked the primary antibody[10].

**Electron microscopic technique:**

At the end of the experiment, the rats were anesthetized using ether inhalation, Perfusion fixation is used, sacrificed, carefully dissected, and adrenal and pituitary glands were fixed in 2.5% glutaraldehyde at 4°C, washed in three to four changes of cacodylate buffer (pH 7.2) for 20 min at every change, and post-fixed in 1% osmium tetroxide for 2 h. They were then dehydrated in ascending grades of ethanol. After immersion in propylene oxide, the specimens were embedded in epoxy resin mixture. These samples were kept in an incubator at 35°C for 1 day, then at 45°C for another day, and finally at 60°C for 3 days. Semithin sections (1-μm-thick) were prepared using an LKB (Bromma, Sweden) ultramicrotome, stained with 1% toluidine blue, and examined by means of a light microscope. Ultrathin sections (500–800 Å) were stained with uranyl acetate and lead citrate and examined using an electron microscope Jeol JEM 1010 (Tokyo, Japan) at 80 kV at an electron microscopic unit, at the Faculty of Medicine, Sohag University, Egypt. [11].

**Histomorphometric and statistical studies:**

1-Thickness of zona fasciculata was measured.

2-The number of corticotrophs positive immunoreactive cells was counted.

3-The number of caspase 3 positive immunoreactive cells was counted.

All measurements were taken using the image analyzer (Leica Q 500 MC program, Wetzlar, Germany) in the Histology Department, Faculty of Medicine, Sohag University, Egypt. Examinations were performed in 5 high-power fields X400/five different sections of each rat. Histological statistical analyses were performed using Paired t test SPSS program, version 17, (IBM Corporation, Somers, New York, USA). P values of < 0.05 regarded as statistically significant.

**RESULTS**

**Adrenal gland:**

**Light microscope of semi thin sections:**

-In group I, zona fasciculata appeared in the form of cords of polygonal cells with well defined cell boundaries. These cells contained rounded vesicular nuclei with prominent nucleoli. The cytoplasm was rich in lipid droplets. The cell cords were separated by sinusoidal capillaries (Fig. 1). In group II, ill defined cell boundaries were observed. Many deeply stained shrunken cells were noted with pyknotic nuclei and few unstained lipid droplets. Other cells appeared with destructed membranes (Fig. 2) In group III, most of the cells had vesicular nuclei and many unstained lipid droplets. Few shrunken deeply stained cells with deeply stained nuclei were observed (Fig. 3).
Immunohistochemical stain:

Anticaspase-3:

In group I, anti caspase-3 immunopositive cells were detected by their brownish cytoplasm and nuclei (Fig. 4).

In group II, there was apparent marked increase in number of anti caspase-3 immunopositive cells (Fig. 5).

In group III, there was apparent mild increase in number of anti caspase-3 immunopositive cells (Fig. 6).

Electron microscopic examination:

Ultrastructurally, in group I, the cells contained numerous lipid droplets, numerous rounded mitochondria and smooth endoplasmic reticulum. The nuclei were euchromatic with prominent nucleoli (Fig. 7). In group II, the cells appeared shrunken with irregular small heterochromatic nuclei. Their electron dense cytoplasm contained fused lipid droplets and lysosomes. (Fig. 8). Other cells had electron dense cytoplasm crowded with numerous lipid droplets (Fig . 9). In group III, most of the cells had euchromatic nuclei with prominent nucleoli (Fig. 10). Numerous mitochondria and lipid droplets were observed.

Pituitary gland:

Light microscope of semi thin sections:

Toluidine blue:

In group I, corticotrophs had rounded nuclei with peripheral arranged small granules (Fig. 11) In group II, some pars distalis cells had deeply stained nuclei and vacuolated cytoplasm. Others had rarified cytoplasm (Fig. 12). In group III pars distalis cells were more or less similar to control apart from some cells with vacuolated cytoplasm. Congested dilated blood vessels were observed (Fig. 13).

Immunohistochemical stains:

Anti ACTH

In group I, anti ACTH immunopositive cells were detected by their brownish granulated cytoplasm (Fig. 14). In group II, there was apparent marked increase in number of anti ACTH immunopositive cells (Fig.,15) .In group III, there was apparent mild increase in number of anti ACTH immunopositive cells (Fig. 16).

Electron microscopic examination:

Ultrastructurally, in group I corticotrophs had euchromatic nuclei. The cytoplasm contained mitochondria, rER and peripherally arranged granules (Fig. 17). In group II, the nuclei were euchromatic. The cytoplasm had prominent Golgi body, mitochondria, destructed criśtae, dilated RER cisternae and numerous variable sized vacuoles. There was apparent decrease in secretory granules (Fig. 18). In group III, corticotrophs appeared more or less similar to the control apart from few vacuoles (Fig. 19).

Statistical results:

There was a significant decrease in the thickness of zona fasciculata in group II in comparison with the control group. There was a significant increase in the number caspase-3 immunopositive cells and ACTH immunopositive cells in group II and group III in comparison with the control group (Table 1) and (Histograms 1- 3).

<table>
<thead>
<tr>
<th>Table 1: Mean ±SEM of the mean thickness of zona fasciculata, caspase-3 immunopositive cells and the number of ACTH cells and of different groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group I</td>
</tr>
<tr>
<td>Mean thickness of zona fasciculata</td>
</tr>
<tr>
<td>Number of caspase-3 positive cells</td>
</tr>
<tr>
<td>Number of ACTH positive cells</td>
</tr>
</tbody>
</table>

P** highly significant ≤ 0.001
* P < 0.01 in comparison with group I
#P < 0.01 in comparison with group II
**Histogram 1:** A histogram showing, mean thickness of zona fasciculata of different groups

**Histogram 2:** A histogram showing, the number of Caspase 3 immunopositive cells of different groups

**Histogram 3:** A histogram showing the number of immunopositive corticotrophs cells of different groups.
Fig. 4: A photomicrograph of immune histochemical staining of caspase-3 in the adrenal gland of control group I showing negative immune reaction. X400.

Fig. 5: A photomicrograph of immunohistochemical staining of caspase-3 in the adrenal gland of group II showing high expression of both nuclear (arrow) and cytoplasmic positive cells (arrowhead). X400.

Fig. 6: A photomicrograph of immunohistochemical staining of caspase-3 in the adrenal gland of group III showing moderate expression of caspase-3 positive cells. X400.

Fig. 7: An electron micrograph of zona fasciculata cells of group I showing euchromatic nucleus with prominent nucleolus (N), numerous mitochondria (M), lipid droplets (LD) and SER (arrow). X600.

Fig. 8: An electron micrograph of zona fasciculata cells of group II showing shrunken apoptotic cell with irregular small heterochromatic nucleus (N); lipid droplets (L) and numerous lysosomes (arrow). X 600.

Fig. 9: An electron micrograph of zona fasciculata of group II showing apoptotic cell has heterochromatic nucleus (N), electron dense cytoplasm crowded with numerous lipid droplets (L). x600.
Fig. 10: An electron micrograph of zona fasciculata cell of group III showing euchromatic nucleus (N), numerous mitochondria (M) and lipid droplets (LD). X600.

Fig. 11: A photomicrograph of a semithin section in pars distalis of group I showing pars distalis cells. Corticotrophs have rounded nuclei and peripheral arranged granules (C) Toluidine blue stain X 1000.× 400.

Fig. 12: A photomicrograph of a semithin section in pars distalis of group II showing some pars distalis cells have deeply stained nuclei and vacuolated cytoplasm (arrow) while others have rarified cytoplasm (*) Toluidine blue stain X 1000.

Fig. 13: A photomicrograph of a semithin section in pars distalis of group III showing pars distalis cells are more or less similar to control © apart from few cells with vacuolated cytoplasm (arrow). Note: congested dilated blood vessels (arrowhead) Toluidine blue stain X 1000.

Fig. 14: A photomicrograph of pars distalis cells of group I showing anti-ACTH immunopositive cells appeared mainly cytoplasmic reaction (arrow). x400.

Fig. 15: A photomicrograph of pars distalis cells of group II showing numerous anti-ACTH immunopositive cells (arrow). x400.
DISCUSSION

Aspartame is hydrolyzed in the intestine to its components; phenylalanine, aspartate and methanol. Each one of these components is toxic to different body organs. About 50% of ASP is composed of phenylalanine which acts as a precursor of catecholamines. While, 40% of ASP is comprised of aspartate (or aspartic acid), which is an excitatory neurotransmitter. Methanol, forming 10% of the broken down products of ASP, is a toxic compound converted in the liver to formaldehyde which is considered carcinogenic [2]. Phenylalanine can follow one of the two pathways of uptake in the body; part is converted into tyrosine (a nonessential amino acid) in the liver by the enzyme phenylalanine hydroxylase [2]. The remaining portion of phenylalanine will bind to a large neutral amino acid transporter to be carried over the blood–brain barrier [12]. Formaldehyde is further broken down into formic acid, which accumulates in the brain, kidneys, spinal fluid, and other organs and can lead to excess acid in body fluids, which is known as acidosis [2].

In the present study aspartame treatment led to apoptotic changes in the zona fasciculata detected by both light and electron microscope and immunohistochchemical stains. Mechanism of apoptosis induced by aspartame is through apoptotic factors such as cytochrome c, apoptosis-inducing factor, and caspase family proteins as aspartame exposure increased the expressions of caspases 8 and 9, so aspartame induced apoptosis mainly via mitochondrial pathway involved in apoptosis due to free radicals [13]. Aspartame led to increased lipid peroxidation products concomitant with depletion of antioxidant parameters. ASP might induce redox and lipid imbalance in rats via mechanism that involves oxidative stress and depletion of glutathione-dependent system. Reactive oxygen species is known to activate nuclear factors which interfere with glucocorticoid-releasing function [14]. In this research, there were increased lipid droplets in apoptotic shrunken cells of
of zona fasciculata. Lipid accumulation was considered a secondary phenomenon due to the inhibition of the sequence of reactions important for biogenesis of steroids[19]. These lipid droplets accumulated in adrenal cortical cells contained much of the cholesterol used in steroid synthesis[10]. These stores of cholesterol in the form of ester represent the cholesterol substrate required for sustained adrenal steroidogenesis[17]. Increase apoptosis of zona fasciculata resulted in decrease in its hormonal secretion. Adrenal hypo function led to positive feedback mechanism which explained the number of corticotrophic cells detected by immunohostaining. Pituitary hyperplasia occurs as a result of adrenal hypo function this led to decreased circulating glucocorticoids with subsequent increased corticotropic releasing factor from hypothalamus. Pituitary cell proliferation is governed by numerous hormonal factors that include hypothalamic releasing hormones and lack of feedback suppression by adrenal hormones. Another mechanism of this hyperplasia may be the activation of cell cycle. The rate of progression "through the cell cycle" is controlled by cell cycle kinases. It was proved that aspartame enhance some growth factors for example vascular endothelial growth factor. Previous studies proved that this factor increases the number of corticotrophs[18], This is another explanation of increased number of corticotrophs in group treated with aspartame. In this study, there were dilated rER, such dilatation indicates increased secretory function of the corticotrophs[19]. In the stivea-treated group, there were minimal changes in both zona fasciculata and pituitary gland compared to the changes induced by those treated with aspartame this might be due rapid elimination of stevia from the body[20]. Oral stevioside was completely degraded into steviol that was the only metabolite in the faeces[21]. Previous studies reported stevia beneficial effects on human health including the antihypertensive, antihyperglycemic, antioxidant, anticariogenic and anti-human rotavirus activities [22]. Besides, Brusick reported that stevia didn’t react directly with DNA or demonstrate genotoxic damage to human. On the contrary, other studies proved harmful effects of stevia attributed to stevioside, which is a steviol derivative induces toxicity through generation of lesions in the chromosomal DNA of mammalian cells[23]. Steviol has also been reported to induce chromosomal breakage and gene mutation in mammalian cells[24].

**CONCLUSION**

Aspartame may be hazardous for the zona fasciculata cortical cells with subsequent adrenal insufficiency which subsequently led to positive feedback on the corticotroph cells. Stevia was of little effect on the pituitary adrenal axis and therefore, is better to be used as sweetener.

**CONFLICT OF INTEREST**

There are no conflicts of interest.

**REFERENCES**


Abdelhaliem and Mohamed


الملخص العربي

دراسة مقارنة لآمان الأسبرتام و الاستيفيا على محور اتصال الغدة النخامية والكظرية في ذكور الجرذ الأبيض: دراسة هستولوجية وكميائية نسيجية مناعية

ضحى صابر أحمد، نسرين جمال الدين عبد الحليم

قسم الهستولوجي، كلية الطب جامعة سوهاج

المقدمة: يعتبر أسبرتام الأوسع استخداما كمنتج صناعي للتحليه. وتعتبر الاستيفيا منتج طبيعي للتحليه له أهمية طبية وتجاربية ويستخدم في معظم دول العالم. ويتم تنظيم الاستجابة للضغط العصبي عن طريق كل من الجهاز العصبي المركزي والضفتي. وتعتبر نواة ما حول البطين المؤثر الأساسي في الاستجابة للضغط العصبي هذا التجمع يسمى محور الاتصال ما بين الغدة النخامية والكظرية والمهاد.

الهدف من البحث: المقارنة بين تأثير الأسبرتام واستفيا على محور اتصال الغدة النخامية والكظرية.

أدوات وطرق البحث: تم تقسيم ثلاثون جرذ إلى ثلاث مجموعات المجموعة الأولى تم استخدامها كمجموعه ضابطه. المجموعة الثانية تم إعطاءها الأسبرتام عن طريق الفم بجرعة 250مج/كجم. المجموعة الثالثة تم إعطاءها الاستفي عن طريق الفم بجرعة 250مج/كجم.

النتائج: لوحظت مظاهر الموت المبرمج في المجموعة التي تلقت الأسبرتام وبالتالي زيادة في عدد الخلايا المفرزه للكورتيزون في الغدة النخامية وكانت التغيرات طفيفة في المجموعة التي تلقت الاستفي بدون زيادة ملحوظة في عدد الخلايا المفرزه للكورتيزون.

الخلاصة: مقارنة بالاسبرتام يعتبر استخدام استفي كمحلي طبيعي أكثر آمنا على محور الاتصال ما بين الغدة النخامية والكظرية.