

EFFECT OF ASPARTAME ON THE RATS' PAROTID SALIVARY GLANDS (LIGHT MICROSCOPIC STUDY)

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ABSTRACT

INTRODUCTION: Aspartame (ASP) is a widely used synthetic sweetener around the world. Its sweetness power is nearly 200 times that of sucrose. It is added to many products, such as diet soda, yogurt, and cereals. Upon long term uptake of ASP, the balance between oxidants and antioxidants is disturbed leading to oxidative stress and peroxidation of lipid resulting in major impacts. Additionally, it causes degenerative changes regarding different body organs.

OBJECTIVES to evaluate the effect of aspartame on the rats' parotid salivary glands.

METHODOLOGY: Sixteen male albino rats (170-210 g in weight) were used in this study. The rats were randomly split into: Group I (control group), Group II (ASP group). Using oral gavage, group I rats were given 1 ml distilled water once a day while group II rats were given 250 mg/kg body weight of ASP dissolved in distilled water once a day throughout the experiment period. After 8 weeks, the animals were euthanized, the parotid glands were surgically removed, and the effect of ASP was evaluated using a light microscope. Acinar surface area was assisted by histomorphometric analysis.

RESULTS: Normal histological features of parotid gland were demonstrated in the control group. Regarding the ASP group, there were changes in the parotid glands' histology. Structural disorganization as well as extensive vacuolation were seen in the serous acini. According to body weight changes, there was an increase in body weight within the ASP group. There was a statistically significant decrease in acinar surface area of group II compared with group I ($p < 0.001$).

CONCLUSION: Oral administration of ASP for 8 weeks in male rats led to significant histological alterations in the parotid salivary glands and thus oral homeostasis disruption.

KEY WORDS: Aspartame, ASP, Parotid salivary glands, Albino rats.

RUNNING TITLE: Effect of aspartame on the parotid salivary glands.

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INTRODUCTION

Artificial sweeteners (AS) are food additives used to sweeten food and drinks. Artificial sweeteners are of two categories; nutritive and non-nutritive based on whether they are a source of calories or not.(1) They are frequently added to foodstuffs and drinks to give them sweet taste without raising their caloric content.(2)

Aspartame (ASP) is considered one of the frequently consumed non-nutritive synthetic sweeteners with a sweetening power of nearly 200 folds that of sucrose. Aspartame (L-Aspartyl-L-phenylalanine methyl ester) is utilized as an alternative to sugar in order to control calories intake in patients suffering from diabetes and obesity. (3, 4)

Annually, over 16 000 tons of aspartame are produced worldwide. Around 200 million individuals use this artificial sweetener. Aspartame is added to various beverages and foodstuffs such as breakfast

cereals, desserts, yoghurt, chewing gum, besides carbonated and powdered soft drinks like diet soda. Also, it is used in pharmaceutical industry as a component of sugar-free cough drops and chewable multi-vitamins. (5)

The Food and Drugs Administration (FDA) has confirmed ASP safety in acceptable daily intake (ADI) 50 mg/ kg, however the Joint FAO/WHO Expert and the Committee on Food Additives (JECFA) have stated that the ADI of ASP is 40 mg/kg.(6, 7)

Despite that, there is some evidence on its correlating side effects at abuse doses as hyperglycemia,(8) neurologic and behavioral disturbances,(9) and nephrotoxicity.(10) Because ASP is found in various products, people might consume doses exceeding those approved by FDA by accident which may lead to serious health complications.(11) Prolonged usage of ASP may lead to several symptoms, such as headaches, nausea, sleeplessness, neurological issues, and vision blur.(12)

In our body, ASP hydrolysis leads to production of aspartic acid, phenylalanine and small amount of methanol by the action of esterases and peptidases enzymes in the intestinal lumen. Methanol, a toxicant, is then oxidized to formaldehyde and again to format.(13)

Aspartame metabolites especially methanol metabolites are the reason for most of ASP adverse effects. These metabolites are associated with production of reactive oxygen species (ROS) like hydrogen peroxide (H₂O₂) as well as superoxide anion (O₂⁻) causing degenerative changes in several organs including kidney, liver, and brain.(14, 15)

In humans, there are 3 pairs of major salivary glands. The parotid glands (PG) are the largest. They secrete a watery secretion rich in proteins such as proline-rich proteins, and enzymes such as amylase. As they produce 25% of total saliva secretion, gland malfunction will lead to xerostomia disrupting oral homeostasis.(16)

Since free radicals are involved in the pathogenesis of ASP induced toxicity, ASP continuous consumption may cause degenerative changes in salivary glands including the PG.

There are few studies of aspartame's alleged harmful effects on salivary glands, particularly in humans or other mammals.

In light of this, the study aims to identify the effects of ASP administration on the histological architecture of the parotid glands.

The study's null hypothesis suggests that there will not be significant difference between the control and study groups.

MATERIALS AND METHODS

Study sample:

Sixteen healthy male albino rats weighing around (170-210 g) were used in this study after gaining the approval of the Research Ethics Committee, Faculty of Dentistry, Alexandria University (IRB No. 00010556 – IORG 0008839). The work was done following the guidelines for the care and use of experimental animals according to Medical Research Institute, Alexandria University from where the animals were gotten, and the work was completed. The animals were kept at room temperature in traditional wire-mesh cages. They were fed a standard rat diet and had free water access.

Chemicals:

1- Aspartame: packed in the form of white powder that was purchased from Pharco Pharmaceuticals, Alexandria, Egypt is water soluble.

Grouping (randomization technique):(17)

Using the website (<http://www.Randomization.com>), the randomization scheme was created. Sixteen rats were randomly split into 2 groups.

Group I (control group): 8 rats were housed in normal condition and given 1 ml distilled water once a day by oral gavage for 8 weeks.

Group II (ASP group): 8 rats were given 250 mg/kg body weight of aspartame dissolved in distilled water once a day by oral gavage for 8 weeks (11)

During the experiment, the rats' body weights were recorded weekly in order to detect any weight changes. After 8 weeks, rats were injected with a lethal dosage of sodium pentobarbital (100 mg/kg) for euthanization. (18)

Histological procedures (19)

After surgical removal of parotid salivary glands, the glands were kept in a 10% neutralized buffered formalin for fixation. Then, the samples were washed, dehydrated in increasing percentages (50%, 70%, 90%, and 95%) of ethanol, cleared with xylene, infiltrated, and embedded in paraffin blocks. Using rotary microtome, the blocks were cut into 5 micrometers thickness sections. After that, sections staining was done using Hematoxylin and Eosin stains then evaluated under a light microscope (BX41; Olympus, Tokyo, Japan) to study their histological architecture.

Histomorphometric analysis

The value of the acinar surface area was measured in pixels at x400 magnification in both groups using the (Image J 1.53) software.

Statistical Analysis

A) Body weight changes in rats.

B) Morphometric measurements of surface area of acini.

Using the Shapiro Wilk test and Q-Q plots, normality was checked. Repeated measures ANOVA was performed followed by multiple pairwise comparisons with a Bonferroni correction to keep the Type I error at 5%. Using Mann Whitney U test, the percentage change was compared. All tests were 2 tailed and the significance level was set p value ≤ 0.05. Utilizing IBM SPSS version 23 for windows, Armonk, NY, USA, the Data were analyzed.

RESULTS

1- Body weight changes results:

Table 1 and Figure 1 illustrate the changes in rats' body weight (g/week) of both groups throughout the experimental period.

Table 1: Comparison between the 2 studied groups in relation to body weights in each week.

	Mean square	F test	p value	Partial Eta Squared
Time	46994.514	43.862	<0.0001*	0.758
Groups	987.007	0.256	0.621	0.018
Time x Groups	411.734	0.384	0.626	0.027

*Statistically significant difference at p value ≤ 0.05

The final body weights of animals increased in both groups after 8 weeks. Rats that received ASP (group II) gained more weight than control rats but there was insignificant difference in mean weights among both groups.

2- Histological results

Group I (control group)

Control group histological specimens revealed parotid gland of normal structure (Figure 2). Regarding the serous acini, they appeared as spherical structures composed of pyramidal cells around a narrow lumen. Prominent spherical deeply stained basally situated nuclei as well as secretory granules filling the apical cytoplasm were observed in the serous cells.

The ducts seen between the acini were of 2 types: intercalated and striated ducts. Due to their compression between the acini, the intercalated ducts were hardly apparent. They were characterized by a lining of cuboidal cells with central nuclei. While the striated ducts lining was columnar cells surrounding wider lumina than the intercalated ducts. The striated ducts' cells showed rounded, darkly stained nuclei and basal striations.

The excretory ducts lined with pseudostratified columnar epithelium were seen in the connective tissue septa separating the gland lobes with some blood vessels (Figure 2).

Group II (ASP 250 mg/Kg)

Disorganized parotid gland tissue was seen in the ASP group histological sections (Figure 3). Regarding the serous cells, their boundaries were ill-defined and excessive vacuolization was noticed in their cytoplasm (Figure 4). Some pyknotic and enlarged nuclei were seen. Also, increased mitotic figures were evident in some acinar cells.

Moreover, some of the striated ducts exhibited slight widening in the lumen in addition to stagnant secretion. Cytoplasmic vacuolations are not only found in acinar cells' cytoplasm but also in the striated ducts cells' cytoplasm. The striated duct cell revealed degradation with ill-defined cell boundaries in addition to loss of the basal striations.

Excretory ducts showed luminal widening with extensive periductal fibrosis. Stagnant secretion was observed in some excretory ducts. The connective tissue septa were thick and infiltrated with inflammatory cells. Regarding blood vessels, they were dilatated and engorged with RBCs (Figure 5).

3- Histomorphometric results

Table 2 and Figure 6 show the comparison between both studied groups according to surface area of acini.

The results showed a highly significant decrease in mean acinar surface area of group II (ASP) when compared with group I (control) where ($P < 0.001$).

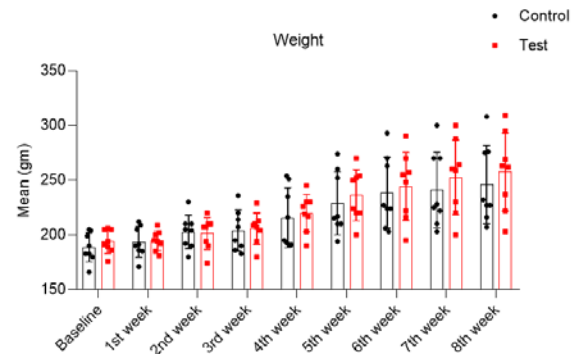


Figure 1: Comparison between 2 studied groups in relation to body weights in each week represented in bar chart with plotted data points.

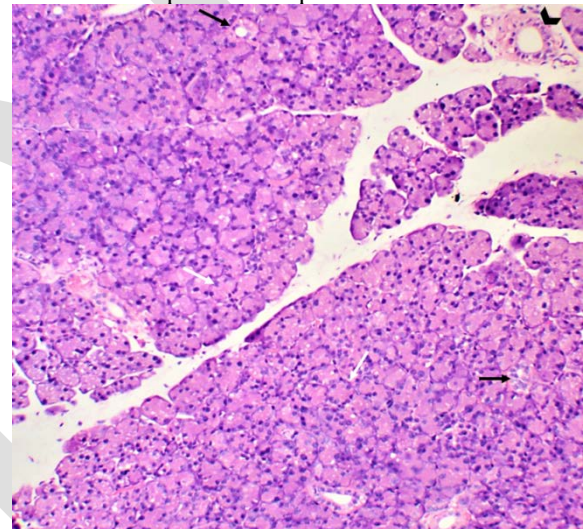


Figure 2: Photomicrograph of control group showing a general view of normal parotid gland structure consisting of serous acini (white arrows), striated ducts (black arrows) and excretory ducts (arrowhead). [H & E stain x100]

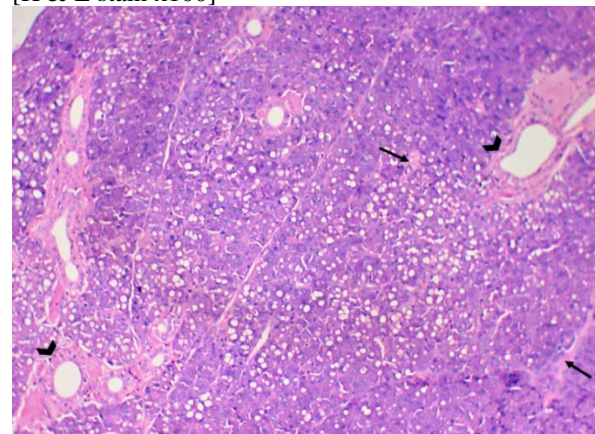


Figure 3: Photomicrograph, (ASP group) showing Disorganization of the acini and duct system architecture. Note the severe vacuolization in acinar cells. Abnormal widening of the ducts (black arrows)

and areas of hemorrhage between the acini (arrowhead) are seen. [H&E stain x 100]

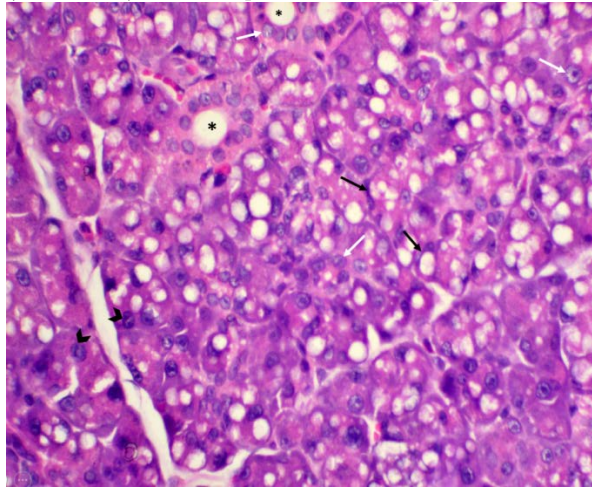


Figure 4: Photomicrograph, (ASP group) showing massive vacuoles in the acini associated with ill-defined borders. Pyknotic (black arrows) and enlarged nuclei (white arrows) are seen. Note the abnormal mitotic figures in some acinar cells (arrowhead) and loss of basal striation of striated ducts (asterisk). [H&E stain x 400]

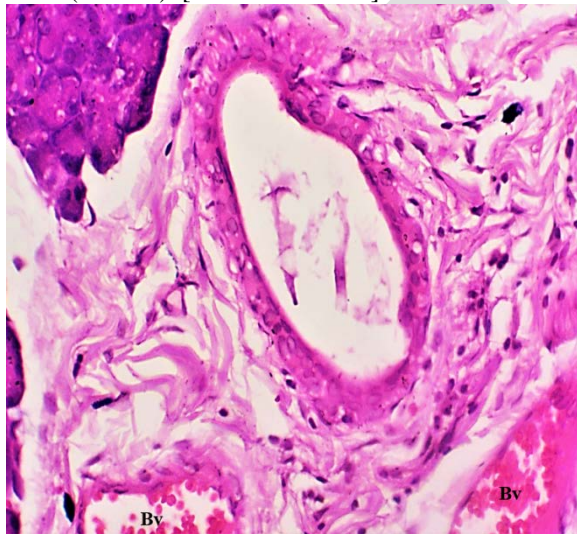


Figure 5: Photomicrograph, (ASP group) showing connective tissue septa between gland lobules exhibiting extensive fibrosis and degeneration with abnormal widening of the excretory duct. Note the stagnant secretion in the duct lumen and the dilated blood vessel (Bv) engorged with RBC's. [H&E stain x 400]

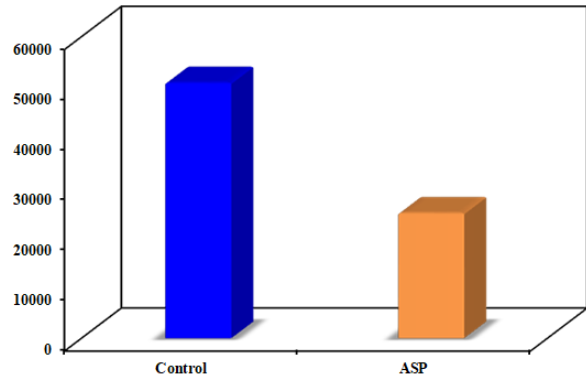


Figure 6: Comparison between the two studied groups according to acinar surface area

Table 2: Comparison between the two studied groups according to acinar surface area

Surface Area	Control (n = 8)	ASP (n = 8)	p value
Min. – Max.	35092.4 – 63548.0	19648.0 – 30890.0	<0.001*
Mean ± SD.	50870.2 ± 9307.7	24948.3 ± 3900.2	

IQR: Inter quartile range SD: Standard deviation
 p: p value for comparing between the two studied groups

*: Statistically significant at $p \leq 0.05$

DISCUSSION

Aspartame is commonly utilized in various products around the world thus its intake is rising daily. Accordingly, additional research is advised to confirm or relieve any concerns people now have about aspartame.

In the digestive tract, ASP is converted into three metabolites; phenylalanine, aspartic acid, besides methanol.(5) The most harmful toxic consequences of ASP are likely caused by methanol oxidation into formaldehyde, and formic acid. It was clearly demonstrated that consuming ASP and the consequent rise in methanol, formaldehyde, and formic acid levels could harm the mitochondrial membrane by producing hydrogen peroxide and superoxide anion, raising ROS level as well as oxidative stress.(20) oxidative stress is thought to be a key factor in cell damage.(21)

In the present study, the parotid salivary glands' response to ASP was evaluated at a dose of 250 mg/kg/d that matched the 40–50 mg/kg/d recommended daily consumption for people as determined by the World Health Organization. Rats metabolize ASP five to six times faster than humans do, hence a dose adjustment for species was necessary.(22)

Regarding the statistical analysis of the rats' body weights, revealed that there was a statistically

insignificant increase in body weights within the ASP group. This insignificant increase in weight is consistent with the work of Khamise NA et al.(23) While an accelerated weight gain was reported in Gul SS et al upon ASP consumption in mice.(24) However, other study stated a loss in weight of rats receiving ASP in comparison to the control group.(25)

The histological findings demonstrated that ASP administration to rats caused several degenerative alterations that were seen in both acini and ducts. Intracytoplasmic vacuoles and undefined cell borders were seen in most acinar cells besides some striated ductal cells. According to another research, spleen of ASP group showed loss of architecture and lymphocytes revealed pyknotic nuclei multiple intracellular vacuoles.(26) These vacuoles were also reported by Mohammed et al. (2015) in submandibular gland and by Omar (2009), who found that frontal cortex pyramidal cells showed substantial cytoplasmic vacuolization and pyknotic nuclei after ASP use at a dose of 250 mg/kg/day for 8 weeks.(15, 27) Omar (2009) concluded that the metabolism of ASP results in the production of several free radicle species that might harm various proteins inside the cell. The granular appearance seen in the acinar and ductal cells after ASP usage demonstrated by this study was in line with Woolf's statement that the first sign of cellular damage is cell expansion brought on by an accumulation of water giving cells a granular appearance.(28)

Stagnant secretion seen in some excretory ducts was also reported in Mohammed et al.(27) The excretory ducts' sluggish secretion may be due to mitochondrial disorders brought on by calcium influx into the cells by the actions of methanol and aspartate. This mitochondrial disorder resulted in ATP depletion, loss of biosynthesis, and membrane pumps, which left the cells without energy for the secretion transport mechanism. (29)

The current Investigation showed thickened connective tissue septa with infiltrated chronic inflammatory cells. Also, dilated blood vessels with engorged RBCS were frequently seen. According to Zahran DH, blood vessels dilation and congestion were also obvious in the lamina propria numerous of the tongue's dorsal surface following prolonged usage of ASP. (11) Congestion of the blood vessels may contribute to inflammation by increasing blood flow to the affected area.(30) According to Alleva et al.(2011), ASP induces oxidative stress, the production of interleukin-6 (IL-6), vascular endothelial growth factor A (VEGFA), and their receptors, besides mitogen-activated protein kinases activation like extracellular signal-regulated kinase (ERK) and p38 MAPK (p38), which favor the

development of inflammatory disorders.(31) Providing the explanation for the thickening of the connective tissue septa in the current experiment, Menezes et al. stated that major harms were mended with collagen fibers or scars regardless of their etiology.(32)

In the current study, aberrant mitosis, pleomorphism, and hyperchromatism of the nuclei of serous cells from ASP group were all indicators of premalignancy. Mohammed et al. also noted analogous precancerous changes in the acinar cells in rats' submandibular glands following daily ASP consumption for four months at a dose of 40 mg/kg.(27)

In conclusion the current findings show that long-term use of ASP might have negative effects on human health, implying the need to reconsider the rules for risk assessments.

CONCLUSION

The results of this study proved ASP toxicity on parotid salivary glands of rats representing in histological changes in parotid glands' structure. Thus, it may as well lead to disturbance in oral homeostasis. It is recommended to pay more attention to ASP usage in various beverages and foodstuffs. The addition of ASP to different food and pharmaceutical products should be restricted.

Conflict of interest

The authors state that they have no conflicts of interest.

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