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Effects of Aspartame on Fetal Kidney: A Morphometric and Stereological Study

Efectos del Aspartame en el Riñón Fetal: Estudios Morfométrico y Estereológico

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SUMMARY: The objective of this study was the evaluation of aspartame effects on morphometric alterations of the glomerulus, proximal and distal convoluted tubules and collecting ducts of the rat fetal kidney during organogenesis. Fifteen pregnant rats averaging 24 g body weight, were divided into 3 groups (n=5 each) of controls, rats treated with aspartame exposed to room temperature and rats treated with aspartame heated to 40ºC. Animals were given 14mg / kg aspartame by the intragastric route on the 9th, 10th, and 11th days of pregnancy. Karyometric and stereological techniques estimated morphological changes. A significant decrease of fetal body weight was observed in the group given aspartame kept at room temperature, compared to controls. Karyometry permitted the estimation of the significant nuclear variations observed in the cells of the glomerulus, proximal and distal convoluted tubules and collecting ducts of aspartame-treated rat fetuses. Stereolgical parameters showed statistically significantly increased cell volume and decreased numerical cell density in fetal kidneys of rats treated with aspartame heated to 40º compared to controls. These results indicate that the use of aspartame leads to alterations in all renal structures studied, suggesting this product's nephrotoxicity.

KEY WORDS: Aspartame; Kidney; Morphometry.

INTRODUCTION

Aspartame is the methylester of N-alpha-aspartyl-L-phenylalanine, a polypeptide widely employed as an artificial, intense synthetic sweetener, having none or insignificant caloric values and a sweeting power higher than that of sucrose. Its use in pharmaceutical products and in nearly 6000 food products, has been approved; among its consumers are those that replace natural foods by "light" ones (AFSSA, 2002).

Aspartame was discovered in 1965; it took 17 years to be approved for consumption by the United States Food and Drug Administration (FDA) in 1981 (Stengik, 1987), and 29 years for its approval by the European Union, in 1994 (ABIAD, 1994). These periods were marked by controversy about its innocuousness that led to requests for toxicological research that still persists at the present time (AIC, 2005).

Several studies on laboratory animals have been made to verify aspartame's toxicity. Recently, a very large experiment confirmed that it is is a multipotential carcinogenic agent when given at a daily dose of 20 mg/kg body weight, an amount well below the acceptable daily dose of 40mg/kg body weight (Sofritti *et al*., 2004).

Since 1976, several studies have discussed the need of comments on two aspects of aspartame metabolism: production of phenylalanine and relationship to phenylacetonuria, and the appearance of methanol leading to the formation of methanolic acid, and extremely toxic compound to our organism at already relatively low amounts (Liesovouri & Heikki,1991; Davoli,1986; Goerss *et al*., 2000).

The increased market of dietary products and the development of new synthetic sweetening compounds have not been sufficiently explored. Thus, to verify the risks and benefits of a substance present in our day-by-day use like aspartame, leads us to worry about the actions of its metabolites (aspartic acid, phenylalanine and methanol) to our organism (Boehm & Bada, 1984; Ishi, 1981).

Although renal excretion is the major process for the removal of toxic substances, renal participation in the

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assimilation of many substances including aspartame, has not been considered.

Bearing in mind their indiscriminate utilization, the understanding of how certain peptides are metabolized by the kidney, may fill in up to now existing gaps in the understanding of their morphometric effects on this organ (Cardello *et al*., 2001).

The aim of the present study was to evaluate the effect of aspartame on the fetal kidney of rats, by observing through karyometry and stereology, structural changes in the glomerulus, proximal and distal convoluted tubules and collecting duct.

MATERIAL AND METHOD

Fifteen albino femaleWistar rats averaging 220-g bodyweight were kept in individual cages and fed a commercial ration and water *ad libitum*. The study protocol was approved by the Ethics Committee for animal experimentation of the Faculty of Medicine of São José do Rio Preto, SP, Brazil. Animals were mated overnight with males at the proportion of 1:4; in the morning their vaginal smears were examined for presence of spermatozoids, and if so, were exposed on the 9^{th}, 10^{th} and 11^{th} day of pregnancy i.e., during the period of organogenesis (68), to a 14 mg/kg body weight dose of aspartame.

On the 20^{th} day of pregnancy, the animals were sacrificed, fetuses collected, immediately immersed in Alfac solution, fixed for 5 days, and weighted on a precision balance. To obtain permanent histological preparations, samples from 30% of the treated and control aleatorily chosen offspring, were obtained. Fetal kidneys were dissected and 6 µm slices cut in semi-serial way, and stabilized with hematoxilin-eosin. Morphometry utilized karyometric and stereological evaluations of fetal kidney glomeruli, convoluted proximal and distal tubules and collector ducts.

Glomerular volumes were estimated using an optical Hund 11500 Wetzlar microscope (Helmut Hund Gmbh, Germany), with a 40x objective, and a Leitz Wetzlar camera lucida, yielding a final 490-fold magnification. Glomerule images were projected on white sulfite paper and their contours drawn with a black, Nº 2 pencil. Twenty structure drawings were obtained from each animal, totalizing 150 for each group.

Karyometry was employed to evaluate nuclear shape and volume as well as their relationships, by averaging the largest and smallest diameters of the epithelial cell nuclei of the proximal and distal convoluted tubules and the renal duct collectors. Slices were analyzed under the above-described microscope with a 100 x objective provided with a Leitz Wetzlar camera lucida, at a final 1240 fold magnification. The contours of the 50 images obtained from each structure were drawn as described above, care being taken to annotate only elliptical images. A millimeter ruler was used to obtain the largest (D) and the smallest (d) axis diameters of the images. These diameters, as well as mean diameters, their ratios, areas, perimeters, volume/area relationships, eccentricity, contour indexes, shape factor and coefficients were evaluated. For stereology, the Merz (Merz, 1968) grid was utilized: slices were focussed under an optical microscope with a 100x objective, and a camera lucida. Images obtained were drawn on the grid. The aim was to obtain cytoplasm volumes, cell volumes, nuclear/cytoplasm relations and numerical density, by counting points remaining superposed to the nuclei and cytoplasm of these cells and on sinusoids of the fetal regions of treated groups, heat-treated groups and controls respectively, totaling the utilization of 20 different fields.

A computer program in Basic Advanced language, developed by Professors Geraldo Maia Campos and Miguel Angel Sala, from the Stomatology Department of the Odontology School of Ribeirão Preto, USP, was utilized. Comparisons between groups were performed by Mood's test for medians, Kruskal-Wallis test, and the Mann-Whitney test, when significant ($P<0.05$) differences had been found.

RESULTS

Results for initial and final body weight parameters.

Results for the quantitative parameters placenta weight and umbilical – cord length for control rat fetuses and those treated with aspartame are shown in Table II.

Glomerulus. It is to be noted that the kidney volume of the kidney of animals given room temperature-treated aspartame and animals given 40ºC-treated, dissolved preparation, were significantly higher than that of the control group (Table III).

Proximal convoluted tubules. Results demonstrated that there was no statistically significant difference between the size of the lesser diameter and the ratio greatest/ smallest diameters, and that there was no modification of the cell shape factor corresponding to the eccentricity, contour index and shape coefficient (Table IV).

Table I. Average values of initial (I) and final (F) body weights (g) of controls animals given at the end of pregnancy, room temperature-treated aspartame, or animals given 40º C-treated, dissolved aspartame, respectively.

Parameter	Control		Room temperature-treated aspartame		40º C-treated, dissolved aspartame	
	Initial	Final	Initial	Final	Initial	Final
Corporal weight(g)	240.7 ± 10	346.5 ± 13	245.1 ± 8	320.1 ± 6*	242.8 ± 10	331.9* ± 11

* p = 0.004 vs. control.

Table II. Average values of placenta weight (g) and umbilical cord length (cm) of control rats fetuses and treated with aspartame.

Parameter	Control	Room temperature treated aspartame	40º C-treated, dissolved aspartame	P
Placenta weight	0.46 ± 7	0.25 ± 10*	0.26 ± 5	0.004*
Umbilical cord length	1.86 ± 6	1.81 ± 8	1.82 ± 10	0.075

* p < 0.05 vs. control.

Table III. Median values of nuclear volumes of the glomerulus, in μm^3, of fetuses from control rats, animals given room temperature-treated aspartame and animals given 40º C-treated, dissolved aspartame, respectively.

Fetuses	Control	Room temperature-treated aspartame	40º C-treated, diluted aspartame
1	1599	2102	1784
2	648	2652	1831
3	1347	1637	2254
4	1396	1793	1994
5	1422	2109	2171
Mediana	1396	2102*	1994*
dIQ	513	665	405

*p < 0.05 vs. control; dIQ –interquartilica diference.

Table IV. Median values of nuclear parameters (n=50) of cells of the proximal convoluted tubules of the fetuses from controls, animals given room temperature-treated aspartame and animals given 40º C-treated, dissolved aspartame, respectively.

Nuclear parameter	Control	Room temperature-treated aspartame	40º C-treated, diluted aspartame	P
Shortest axis	7.66±1.94	8.22±0.14	8.44±1,71	0.082
Geometric mean axis	8.36±1.82	9.06±0.42	10.75±1,42*	0.006*
Ratio D/d	1.22±0.37	1.15±0.14	1.22±0,37	0.343
Volume (μm^3)	341±182	428±212	685±122*	0.006*
Área (μm^2)	56.9±22.3	66.6±10.9*	68.0±16,8*	0.031*
Perimeter (μm)	26.5±5.3	28.6±2.6*	30.5±14,3*	0.006*
Ratio V/A	5.58±1.21	6.04±0.28*	6.54±1,55*	0.031*
Eccentricity	0.52±0.16	0.45±0.07	0.54±0,14	0.435
Shape factor	0.98±0.01	0.99±0.01	0.86±0,20	0.153
Countor index	3.58±0.06	3.56±0.04	3.45±1,05	0.153

Mediana ± interquartilica diference; * p < 0.05 vs. control.

Distal convoluted tubules showed increased nuclear diameters, implying an increased perimeter, area and volume of these nuclei in fetuses from both groups of treated rats. The shape of these nuclei (eccentricity, shape coefficient and contour index), presented a significant alterations in animals given room temperature-treated aspartame (Table V).

Collecting ducts revealed significant variations of parameters like the greater diameter, medium diameter, area, perimeter and volume/area ratio in the group given 40º C-treated, dissolved aspartame. In both treated groups, volumes increased significantly, but there was no significant variation of nuclear shapes compared to controls (Table VI).

Table V. Median values of nuclear parameters (n=50) of cells of the distal convoluted tubules of the fetuses from controls, animals given room temperature-treated aspartame and animals given 40º C-treated, dissolved aspartame, respectively.

Nuclear parameter	Control	Room temperature-treated aspartame	40º C-treated, dissolved aspartame	P
Longest axis	7.15±0.88	10.80±1.55*	12.98±1.33*	0.006*
Shortest axis	5.34±0.65	8.42±1.29*	8.92±4.12*	0.031*
Geometric mean axis	6.15±0.70	9.82±1.74*	10.85±1.50*	0.006*
Ratio D/d	1.40±0.11	1.67±0.20*	1.58±0.17*	0.031*
Volume (μm^3)	128±22	413±106*	633±189*	0.006*
Area (μm^2)	30.2±6.3	70.6±3.8*	64.2±6.1*	0.006*
Perímeter(μm)	19.8±2.3	30.5±3.3*	30.7±7.0*	0.031*
Ratio V/A	4.10±0.47	6.14±0.57*	6.94±2.08*	0.031*
Eccentricity	0.64±0.05	0.74±0.15*	0.55±0.15	0.003*
Shape factor	0.95±0.02	0.82±0.13*	0.86±0.11	0.003*
Countor index	3.63±0.04	3.72±0.07*	3.68±0.56	0.031*

Mediana ± interquartilica diference ; * $p < 0.05$ vs. control.

Table VI. Median values of nuclear parameters (n=50) of cells of the collecting ducts of the fetuses from controls, animals given room temperature-treated aspartame and animals given 40º C-treated, dissolved aspartame, respectively.

Nuclear parameter	Control	Room temperature-treated aspartame	40º C-treated, diluted aspartame	P
Longest axis	7.84±1.57	8.45±0.51	10.89±2.14*	0.006*
Shortest axis	5.94±1.26	7.42±0.51	7.97±0.36	0.060
Geometric mean axis	6.44±1.44	7.59±0.67	9.53±1.37*	0.006*
Ratio D/d	1.45±0,29	1,30±0,13	1,55±0,29	0.343
Volume (μm^3)	151±115	332±63*	489±168*	0.006*
Area (μm^2)	33.5±15.8	43.5±10.3	73.0±13.5*	0.006*
Perímeter (μm)	21±4.3	25.7±5.3	34.1±5.2*	0,006*
Ratio V/A	4.29±0.96	5.39±0.70	7.15±0.60*	0.006*
Eccentricity	0.62±0.21	0.55±0.07	0.47±0.44	0.153
Shape factor	0.94±0.05	0.97±0.04	0.93±0.06	0.453
Countor index	3.66±0.11	3.61±0.14	3.68±0.34	0.765

Mediana ± interquartilica diference ; * $p < 0.05$ vs. control.

Stereology. The data shown on Table VII, present the increase of cell and cytoplasm volumes of aspartame-treated groups. On cell quantification, a significant reduction of the proximal convoluted as well as disappearance of many of these structures at the brush border of 40ºC-treated, diluted aspartame rat fetuses. At the distal convoluted tubules, cell nuclei also were found in lesser numbers.

The glomeruli of the treated groups also showed all their stereological parameters to be altered, with larger, but a reduced number of epithelial cells. Nevertheless, the collection ducts did not present statistical differences regarding their numerical density.

Table VII. Overall picture of the medians of stereological parameters of glomerulus, proximal convoluted tubule, distal convoluted tubule and collection duct of rat fetuses of controls, of animals given room temperature-treated aspartame and of animals given 40º C-treated, dissolved aspartame, respectively.

Stereological parameter		Control	Room temperature-treated aspartame	40º C-treated, diluted aspartame	P
Cell volume	Glomerulus	5369.3	5924.3*	6156.7*	0.007*
	P. C. tubule	2118.7	2564.3	2956.4*	0.006*
	D. C. tubule	1965.3	2489.2*	2372.0*	0.026*
	Collection duct	1756.3	1935.5*	2235.4*	0.006*
Cytoplasm volume	Glomerulus	6505.7	6607.8*	6955.0*	0.007*
	P. C. tubule	3897.3	4122.0	4625.3*	0.006*
	D. C. tubule	2565.3	2935.6 *	3024.2*	0.030*
	Collection duct	1854.3	2165.4*	2563.2*	0.007*
Ratio nuclei/ cytoplasm.	Glomerulus	0.5437	0.5568*	0.7533*	0.007*
	P. C. tubule	0.1574	0.1823	0.1960	0.69
	D. C.tubule	0.1355	0.1412*	0.1563*	0.006*
	Collection duct	0.1023	0.1255*	0.1333*	0.007*
Cell numerical density	Glomerulus	245425	150588 *	148967*	0.007*
	P. C. tubule	150588	165598	130566*	0.007*
	D. C. tubule	132564	125658*	112500 *	0.026*
	Collection duct	112367	125438	1095433	0.082

Mediana ± interquartilica diference ; * $p < 0.05$ vs. control.

DISCUSSION

The present study showed that daily orogastric administration of 14mg/kg of aspartame on the 9th, 10th and 11th day of pregnancy, led to alterations in the development of renal structures.

Limited studies had been performed and published by others, who administered aspartame from the 7th to the 14th day of pregnancy, an important period for organogenesis in the rat (Sturtevant, 1985; Holder, 1989; Lennon *et al*.,1980).

In the present experiments, we observed morphological alterations that revealed that aspartame crossed the placenta causing a reduction of fetal weight, following the administration, when kept at room temperature or when diluted and heated to 40ºC .This result agrees with those by others that administered 20mg aspartame /kg body weight daily from the 10th to the 14th day of pregnancy and detected delayed fetal development. Our study suggests that administration of aspartame on the 9th, 10th and 11th day of pregnancy delays fetal growth as expressed by cell damage during this period.

These findings demonstrate the usefulness of the animal model employed for the study of the effects of aspartame on the developing kidney by karyometry and stereology quantification, leading to the finding that there occurs an alteration of kidney structures during pregnancy.

Our observations that aspartame toxicity became more pronounced following its dilution and heating to 40º C, also agreed with those of Tsang (1985) who also used the experimental model. Their study reports that any form of heating of aspartame, rapidly causes the formation of diketopiperazine (DKP) and free phenylalanine. Significant amounts of DKP are also formed when aspartame is stored

in solution at room temperature, but heating considerably hastens this process.

It appears therefore that further work aimed at the elucidation of the mechanisms by which this artificial sweetener determines nuclear and cell alterations in the kidney, is needed.

Based on our results it is to be concluded that karyometry and stereology analyses of groups of fetal rats, suggests nephrotoxicity to the glomerulus, proximal and distal convoluted tubules and to a lesser degree, the collecting ducts, of aspartame kept at room temperature or diluted and heated to 40º C.

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RESUMEN: El objetivo de este estudio fue evaluar las alteraciones morfológicas del aspartame en glomérulos, túbulos contorneados distal y proximal y ductos colectores, en riñones de ratas, durante la organogénesis. Se utilizaron ratas preñadas con un peso promedio de 24g, las que fueron separadas en 3 grupos (n=5 cada uno): un grupo control, otro grupo con ratas tratadas con aspartame y expuestas a temperatura ambiente y un grupo de ratas tratadas con aspartame y mantenidas a 40º C. A los animales se les administró 14 mg/kg de aspartame vía intragástrica en las semanas 9, 10 y 11 de preñez. Con técnicas cariométricas y estereológicas se estimaron los cambios morfológicos. Una disminución significativa del peso fetal se observó en el grupo que recibió aspartame y mantenida a temperatura ambiente, comparado con los controles. La cariometría permitió la estimación de significativas variaciones nucleares observadas en las células de los glomérulos, túbulos contorneados distal y proximal y ductos colectores, en los fetos de las ratas tratadas con aspartame. Los parámetros estereológicos mostraron un incremento estadísticamente significativo del volumen celular y una disminución en la densidad de número de las células, en riñones fetales de ratas tratadas con aspartame y mantenidas a 40º C comparado con los controles. Estos resultados indican que el uso del aspartame lleva consigo alteraciones en todas las estructuras renales estudiadas, lo que sugiere la nefrotoxicidad del producto.

PALABRAS CLAVE: Aspartame; Riñon; Morfometría.

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