WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 9, Issue 1, 24-47. Research Article ISSN 2277– 7105

GLYCATION BY GLUCOSE AND KETONE BODIES OF AMINOPHOSPHOLIPIDS FROM HUMAN TISSUES IS INHIBITED BY L-ARGININE AND CREATINE. II. AORTA ARTERY

José D. Méndez*[a], Adolfo Chávez-Negrete[b] and Guillermo Valero[c]

[a]Medical Research Unit in Metabolic Diseases, Cardiology Hospital. National Medical Center, Instituto Mexicano del Seguro Social, 06703 Mexico City, Mexico.

[b]Education and Research Department. National Medical Center, Instituto Mexicano del Seguro Social. Mexico City, México.

[c]Pathology Department, Cardiology Hospital. National Medical Center, Instituto Mexicano del Seguro Social. México City, Mexico.

Article Received on 22 Oct. 2019,
Revised on 12 Nov. 2019,
Accepted on 02 Dec. 2019,
DOI: 10.20959/wjpr20201-16400

***Corresponding Author**
Dr. José D. Méndez
Medical Research Unit in Metabolic Diseases. Cardiology Hospital. Instituto Mexicano del Seguro Social. Mexico City, Mexico.

ABSTRACT

β-Hydroxybutyrate and acetoacetate are an alternative source of energy for several tissues and organs including the heart. However, large quantities are found in the blood in type 1 and type 2 diabetics with poor metabolic control. High concentrations of ketone bodies are associated with diabetic ketoacidosis. The involvement of ketone bodies in the glycation of bovine hemoglobin and rat brain aminophospholipids has been demonstrated. In this study the effect of L-arginine and creatine on the glycation of human aortic aminophospholipids by glucose and ketone bodies was evaluated. Aminophospholipids were isolated and their purity confirmed by thin layer chromatography. Emulsions were prepared in 0.1 M phosphate buffer, pH 7.4 containing 11 mM glucose, 3 mM β-hydroxybutyrate or 3 mM acetoacetate, respectively. When the effect of L-arginine or creatine was tested, 10 mM concentration was used. Emulsions were prepared and incubated at 37 °C in dark during 61 days. Samples were taken on days 0, 12, 19, 26, 48 and 61. Then, they were analyzed by thin layer chromatography, fluorescence and ultraviolet spectroscopy. Three spots with R_f of 0.12, 0.17, and 0.244, respectively, showed higher intensity. However, several spots with lower intensity were also revealed. Glycation of aminophospholipids was clear. It was higher for glucose followed by β-hydroxybutyrate and acetoacetate. Seen by three ways; thin layer

chromatography, fluorescence and ultraviolet spectroscopy, both L-arginine and creatine affected glycation process. The effect of L-arginine was clear in mixtures containing aminophospholipids, glucose and β-hydroxybutyrate. Less relative fluorescence was observed when creatine was added to mixtures containing acetoacetate. The spectral signals in the ultraviolet region (290 to 200 nm) suggest the formation of several compounds between aminophospholipids, glucose and ketone bodies. In conclusion, these results provide evidence that aortic aminophospholipids are affected not only by high concentrations of glucose but also by ketone bodies. Since glycation has been associated with the development of cardiovascular disease, this process can be prevented with L-arginine and creatine.

KEYWORDS: Acetoacetate, Aminophospholipids, L-Arginine, Creatine, Glycation, Human aorta, β- Hydroxybutyrate, Hyperglycemia.

1. INTRODUCTION

The non-enzymatic reaction of saccharides with proteins, nucleotides and lipids is termed glycation.[1, 2] The products formed by glycation are known as advanced glycation end products (AGEs).[3, 4] The AGEs formation occurs in three stages. In the first stage of glycation, the carbonyl group either aldehyde or ketone group of the carbohydrate attaches to the amino group to produce a carbon-nitrogen double bond structure known as the Schiff base, then an enamine is yielded from the Schiff base and later converts to a ketoamine via ketoenol tautomerism, this process is named the Amadori rearrangement, and the ketoamine product is also called the Amadori product.[5] Under physiological conditions, levels of Schiff base and Amadori products reach equilibrium after 4 weeks. The plasmatic concentration of Amadori compound in equilibrium is around 140 µM in normal individuals, and may increase under diabetes condition.[6] In the second stage, products from the first stage are converted into intermediates such as α-dicarbonyls or oxoaldehydes,[7] which are comprised of derivates of reducing sugars, aldehydes, α-ketoaldehydes, and cyclic structures.[8] Most of the reactions at second stage occur under oxidative conditions.[9] Finally, in the third stage of glycation, reactions between secondary intermediates and specific amino acids of a protein can lead to the yield of acyclic products. Molecules obtained after the third stage of glycation are known as AGEs.[10] Glycated proteins, in particular HbA1c, has been used clinically as molecular markers of hyperglycemia.[11] Recently was informed that higher HbA1c variability is associated with increased risks of all-cause mortality, cardiovascular events, and microvascular complications of diabetes independently of high HbA1c.[12] AGEs activate cell

membrane receptors and regulate pre-inflammatory pathways, which have a great impact in diabetes and related diseases.[13]

Initially, the AGEs formation was studied considering reducing sugars as sources of carbonyl groups. Later, it was discovered that several glycation products such as N^{ε}-carboxymethyllysine can be formed from carbonyl groups derived not only from carbohydrates but also from lipid peroxidation and having the same chemical structure.[14] Today it is known that other aldehydes such as acrolein are formed by the oxidation of polyunsaturated fatty acids.[15] but also by the degradation of polyamines.[16,17] Three main groups of AGEs have been described: 1. Fluorescent crosslinking AGEs. 2. Non-fluorescent crosslinking AGEs. 3. Non-crosslinking AGEs.[18-21]

Other group of carbonyl compounds named ketone bodies; β-hydroxybutyrate, acetoacetate and acetone, has been studied mainly as an alternative source of energy for brain, liver, skeletal muscle, kidney cortex, adipose cells and heart under insulin deficiency.[22] However, large quantities have been found in the blood of individuals who are experiencing diabetic ketoacidosis,[23,24] a complication of diabetes mellitus.

Previously, we informed that hemoglobin can be glycated by acetone and β-hydroxybutyrate forming fluorescent AGEs[25] and that rat brain aminophospholipids are also glycated by acetoacetate.[26] Here, we provide evidence that the aminophospholipids of the human aortic artery are glycated by β-hydroxybutyrate and acetoacetate as do it glucose. In addition, we show how this process can be prevented by L-arginine and creatine.

2. MATERIAL AND METHODS

2.1. Chemicals

Acetoacetate (Lithium salt), β-Hydroxybutyrate, Glucose, Creatine and Ninhydrine were purchased form Sigma (Sigma Chemical Co. St. Louis, MO, USA). L-Arginine and silica gel plates (Cat. 5721 DC-Fertigplatten Kieselgel 60) were purchased from Merck (E. Merck, Darmstadt, Germany). Other reagents were of analytical grade.

2.2. Biological Material

2.2.1. Aortic arteries

Four *post mortem* aortic arteries (16.0 - 29.5 g wet weight ascending aorta) carefully dissected by experimental surgeons were donated by the Pathology Department, Cardiology Hospital. Instituto Mexicano del Seguro Social. Being a research project that only requires

one type of material biological, aortic artery, the confidentiality of the *post-mortem* heart donor was assured and reference was made only to the sample with the registration number given by the Department of Pathology. The inclusion criteria were the following: 1. Samples of non-diabetic subjects. 2. Indistinct sex. 3. Age of donors: Between 20 and 70 years of age. The exclusion criteria were: 1. Samples of diabetic subjects. 2. Age of donors: Under 20 years and over 70 years. The project was authorized by the Ethics Committee of the Siglo XXI National Medical Center for the use of biological material.

2.3. Experimental Procedure

2.3.1. Extraction of aminophospholipids

Aminophospholipids were extracted from aortic arteries following a selective extraction. First, in order to eliminate cholesterol, the aortic tissue was finely cut and crushed in a mortar using acetone in a ratio of 1: 6 (weight / volume). Then transferred and homogenized in a POBEL glass plunger homogenizer for manual laboratory work. The aortic tissue was homogenized and the homogenate was allowed to stand with occasional agitation. It was then vacuum filtered using a buchner funnel and filter paper. The supernatant containing cholesterol was stored in a beaker and the precipitate was re-extracted twice more. The cholesterol fraction was not used in the present study and was discarded. The precipitate was treated with ether (6 ml / g of tissue) for the extraction of aminophospholipids. The residue was re-suspended and allowed to stand for 10 minutes with occasional agitation. It was then filtered under vacuum separating the supernatant fraction. The residue was re-extracted twice more and the supernatants were pooled. The ether was evaporated in a water bath at 37 ^{o}C. The crystals obtained constituted the fraction of aminophospholipids that was used in this study. Purity of aminophospholipids was confirmed by thin layer chromatography as describe below. Lipid fraction was preserved under refrigeration protected from light until be used.

2.3.2. Analysis of aminophospholipids by thin layer chromatography

Four µL of a solution of 1 mg of aorta aminophospholipids dissolved in 1 mL of ether were placed in a silica plate and eluted with a mixture of chloroform-methanol-acetic acid-water (65: 25: 8: 2). The presence of aminophospholipids was visualized using 0.02 % ninhydrin in acetone and heating at 80 °C.[27] (See Figure 1).

2.3.3. Analysis of the purity of L-arginine and creatine

Purity of both L-arginine and creatine was analyzed as described in Part 1. Briefly, solutions of 1 mg / mL containing L-arginine or creatine were prepared in phosphate buffer 0.1 M pH =

7.4. Then, 4 µL were placed in a silica plate. Chromatography was run and developed as described above for aminophospholipids.

2.3.4. Experimental assays

2.3.4.1. Incubation of aminophospholipids with glucose, β-hydroxybutyrate, acetoacetate, L-arginine and creatine

In order to compare the effect of glucose, β-hydroxybutirate and acetoacetate on aminophospholipid from aorta, 3 mM β-hydroxybutyrate, 3 mM acetoacetate and 11 mM glucose were used. When the effect of creatine or L-arginine was tested, 10 mM solutions were prepared. The concentration of aminophospholipids was 68.5 mg in each case. All the substances were mixed in 10 ml of 0.1 M phosphate buffer solution, pH = 7.4. The order of vials was as follow: Vial 1. Aminophospholipids (Control). Vial 2. Aminophospholipids + glucose. Vial 3. Aminophospholipids + β-hydroxybutyrate. Vial 4. Aminophospholipids + acetoacetate. Vial 5. Aminophospholipids + glucose + L-arginine. Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Vial 7. Aminophospholipids + acetoacetate + L-arginine. Vial 8. Aminophospholipids + glucose + creatine. Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Vial 10. Aminophospholipids + acetoacetate + creatine. Duplicate vials were made in all cases. The vials were vigorously shaked. Then emulsified lipids were incubated at 37 ºC in dark conditions for 61 days.

A 500 µl aliquot was taken in duplicate at 0, 12, 19, 26, 48 and 61 days from each vial. Samples were analyzed by thin layer chromatography, fluorescence (Fluoroskan fluorometer ASCENT FL-2.2) and ultraviolet spectroscopy (Lambda 20 spectrophotometer Perkin-Elmer 101N82926). Fluorescence was registered into two ranks of excitation and emission; 320 / 460, and 355 / 460 nm, respectively. The version of the program used by the flourometer was the Ascent Software 2.4.1. In the case of ultraviolet spectroscopy, the scanning spectrum was recorded from 400 to 200 nm.

2.4. Statistical Analysis

Results of fluorescence were analyzed by one-way ANOVA and Dunnett's test.[28]

3. RESULTS

3.1. Thin layer chromatography of aminophospholipids

The results of the chromatographic analysis of the aminosphospholipid extracts are presented in Figure 1. Greater diversity of aminophospholipids was observed in the aortic tissue when

compared to the fraction of aminophospholipids isolated from the erythrocyte membrane (see Part 1). Some spots that were revealed with greater intensity are indicated by the acronym AP1-AP3, whose R_f values were 0.12, 0.17 and 0.244, respectively.

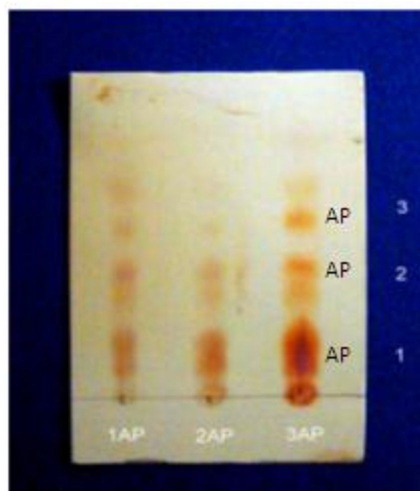

Figure 1: Thin layer chromatography of aminophospholipids obtained from human aorta. The main spots revealed with greater intensity are indicated (AP1-AP3). Other spots showing lower intensity are seen. The samples were applied in triplicate (1AP-3AP).

3.2. Purity of L-arginine and creatine

L-arginine showed two spots; one of higher intensity with a R_f value of 0.3182 and another with very slight intensity, almost negligible, with a R_f value of 0.6136 was discarded for the purposes of this study. Creatine only showed a spot with R_f value of 0.1932 (See Figure 1. Part 1).

3.3. Analysis of the aminophospholipid reaction mixtures

3.3.1. Thin layer chromatography

The results of the sampling performed at days 0, 12, 19, 26, 48 and 61 of incubation are summarized in Figure 2 and Table 1. Among the most important changes are the interaction of glucose, β-hydroxybutyrate and acetoacetate with the aminophospholipids (vials 5, 6 and 7, respectively) and the effect of L-arginine and creatine (vials 7-9) seen at 61 days (Figure 2, right side). This change could be observed from day 12 of incubation (image not shown), but the intensity of the spots was greater as the incubation time increased. Since ninhydrin reacts with free amino groups, several aminophospholipids may not be revealed after the reaction with glucose or ketone bodies.

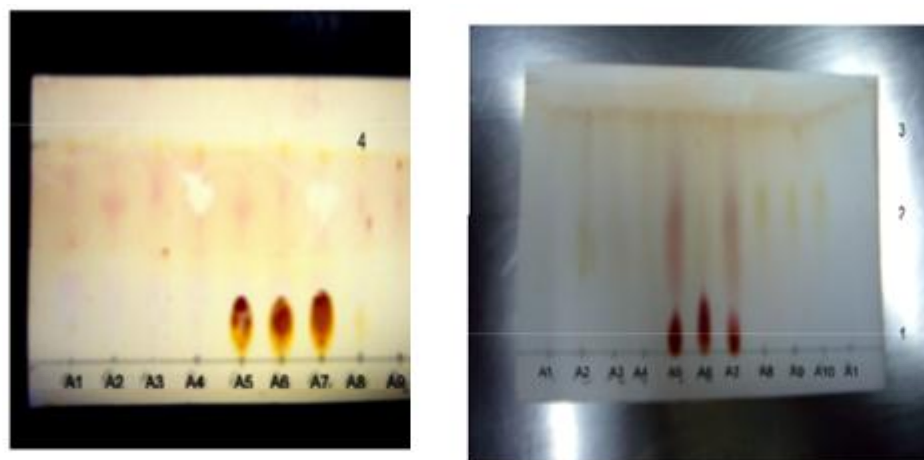

Figure 2: Thin layer chromatography of mixtures of aminophospholipids (AP1-AP3 shown in Figure 1) of aorta with glucose or ketone bodies in the presence or absence of L-arginine or creatine (Left, Day 0 and Right, Day 61. A1-A10 = Vial 1-Vial 10. (See the foot note in Table 1.).

Table 1: R_f values of aminophospholipids of aorta separated by thin layer chromatography at Day 0 and at Day 61 of incubation in different experimental conditions.

Vial No.	Day 0			Day 61		
	AP1	AP2	AP3	AP1	AP2	AP3
1	0.170	0.660	0.840	-	-	0.775
2	0.170	0.739	-	-	0.316	0.755
3	0.173	0.470	0.815	-	-	0.755
4	0.210	-	-	-	-	0.755
5	0.152	0.315	0.629	0.082	0.306	0.776
6	0.152	0.283	0.826	0.102	-	0.776
7	0.163	0.348	-	0.092	0.337	0.776
8	0.140	0.315	0.728	-	0.418	0.796
9	-	0.260	0.780	-	0.429	0.796
10	-	0.320	0.520	-	0.469	0.816

Vial 1. Aminophospholipids (Control). Vial 2. Aminophospholipids + glucose. Vial 3. Aminophospholipids + β-hydroxybutyrate. Vial 4. Aminophospholipids + acetoacetate. Vial 5. Aminophospholipids + glucose + L-arginine. Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Vial 7. Aminophospholipids + acetoacetate + L-arginine. Vial 8. Aminophospholipids + glucose + creatine. Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Vial 10. Aminophospholipids + acetoacetate + creatine. AP=Aminophospholipid.

3.3.2. Fluorescence Analysis

Results of fluorescence analysis registered into two ranks of excitation and emission; 320 / 460, and 355 /460 nm, respectively, are summarized in Table 2. In both emission and excitation ranges the samples showed greater fluorescence intensity on day 12 of incubation including the control sample that only contained aminophospholipids, after which a plateau was reached. In successive sampling fluorescence decreased slightly. It was observed that the reaction mixture containing glucose showed greater fluorescence intensity.

Table 2: Results of the fluorescence analysis of the sampling performed during the incubation time of aortic aminophospholipids mixed with glucose, ketone bodies, L-arginine and creatine.

Vial No.	Wavelengh (320 - 460 nm)						Wavelengh (355 – 460 nm)					
	Day						Day					
	0	12	19	26	48	61	0	12	19	26	48	61
1	582.5	1181.0	1573.0	1519.5	1308.0	1309.5	86.5	129.4	209.3	180.9	170.2	173.4
2	744.5	1516.0	1502.0	1417.0	1400.0	1434.0	103.1	148.9	178.2	169.1	168.4	169.7
3	665.5	1523.0	1451.0	1402.0	1391.0	1433.5	107.1	162.4	173.4	164.3	165.6	169.6
4	610.1	1403.5	1368.5	1300.0	1336.0	1348.5	96.7	150.6	160.7	149.7	154.4	154.5
5	636.1	1493.5	1462.0	1352.0	1474.5	1499.0*	106.8	176.0	187.8	179.0	195.3	201.3
6	733.3	1483.0	1481.5	1405.5	1419.0	1451.0*	122.4	171.0	184.8	181.1	188.6	191.3
7	676.9	1512.5	1470.5	1408.0	1475.0	1499.5*	113.1	180.8	189.3	185.0	196.4	202.8
8	684.7	1315.0	1334.0	1264.5	1275.0	1297.0	110.6	144.4	154.8	147.6	152.8	153.2
9	837.5	1400.0	1353.0	1249.5	1253.0	1282.5	117.8	158.5	160.3	147.9	157.1	163.3
10	699.4	1201.5	1330.5	1234.0	1194.0	1197.5	111.6	134.1	157.0	146.1	147.8	148.4

Vial 1. Aminophospholipids (Control). Vial 2. Aminophospholipids + glucose. Vial 3. Aminophospholipids + β-hydroxybutyrate. Vial 4. Aminophospholipids + acetoacetate. Vial 5. Aminophospholipids + glucose + L-arginine. Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Vial 7. Aminophospholipids + acetoacetate + L-arginine. Vial 8. Aminophospholipids + glucose + creatine. Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Vial 10. Aminophospholipids + acetoacetate + creatine. Relative fluorescence (a.u). The values are the average of the values recorded in both measurements. *($P < 0.05$).

When compared to the control, mixtures containing β-hydroxybutyrate and acetoacetate also showed higher fluorescence. The addition of L-arginine caused an increase in the fluorescence with any of the glycant agents added, probably due to the reactivity of the guanidino group, however the system containing β-hydroxybutyrate showed less

fluorescence. When creatine was added the fluorescence was lower for acetoacetate although the difference with L-arginine is not noticeable.

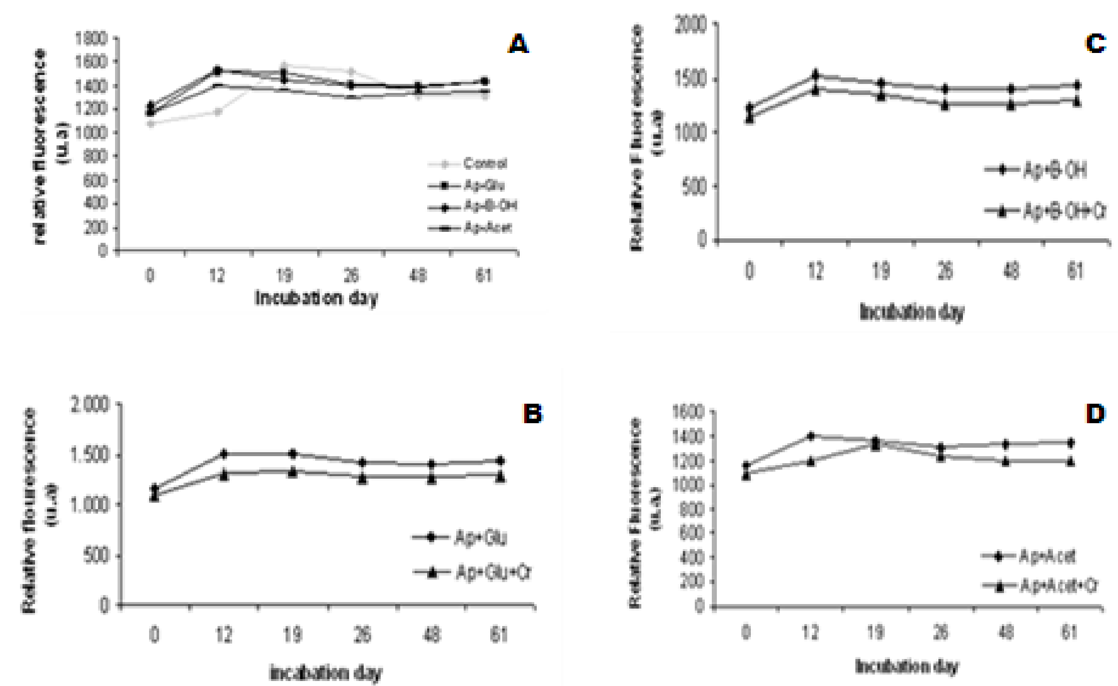

Figure 3: Fluorescence of aminophospholipids incubated for 61 days with glucose, ketone bodies and creatine (360 nm excitation / 460 nm emission). A. Vial 1. Aminophospholipids (Control). An increase in fluorescence can be seen at days 12 to 48 of incubation .Vial 2. Aminophospholipids + glucose (Ap + Glu). Vial 3. Aminophospholipids + β-hydroxybutyrate (Ap + B-OH). Vial 4. Aminophospholipids + acetoacetate (Ap + Acet). B. Vial 2 (Ap + Glu), Vial 8. Aminophospholipids + glucose + creatine (Ap + Glu + Cr). C. Vial 3 (Ap + B-OH), Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine (Ap + B-OH + Cr). D. Vial 4 (Ap + Acet), Vial 10. Aminophospholipids + acetoacetate + creatine (Ap + Acet + Cr).

3.3.3. Ultraviolet spectroscopy

At the end of the incubation time (61 days) the different reactive systems were analyzed by ultraviolet spectroscopy in a scanning interval of 400-200 nm.

3.3.3.1. Aminophospholipids, glucose, β-hydroxybutyrate and acetoacetate

The spectra shown in Figure 4 show the formation of reaction products between aminophospholipids, glucose and ketone bodies. The spectra are similar between glucose, β-

hydroxybutyrate and acetoacetate. It is striking that the sample used as a control also shows some changes in absorption between 300 and 200 nm. The addition of glucose or ketone bodies to the aminophospholipids caused not only an increase in absorption but also in the number of peaks mainly in the region of 290 to 200 nm. It is further observed that the system containing β-hydroxybutyrate exhibited greater absorbance in the region of 300 to 240 nm compared to systems containing glucose and acetoactate.

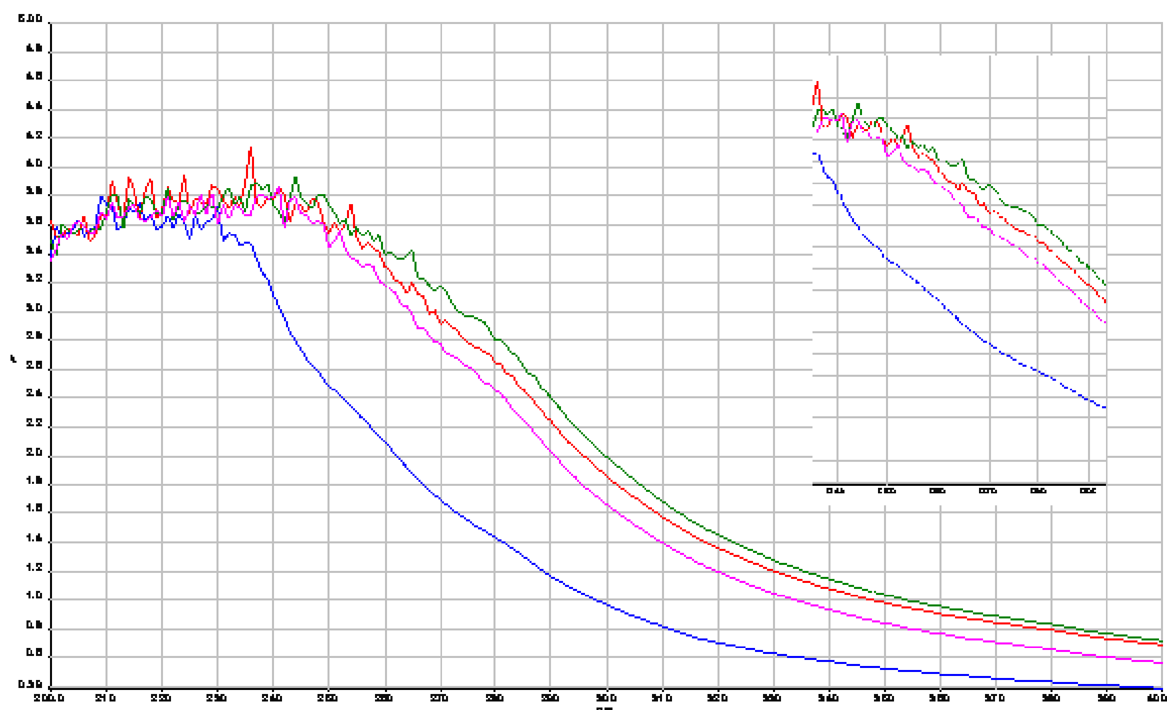

Figure 4: Scanning spectrum 400 to 200 nm (from right to left) of aminophospholipids at day 61 of incubation (Each division corresponds to 10 nm) (Insert: 290-240 nm).Color blue: Vial 1. Aminophospholipids (Control). Red color: Vial 2. Aminophospholipids + glucose. Green color: Vial 3. Aminophospholipids + β-hydroxybutyrate. Pink color: Vial 4. Aminophospholipids + acetoacetate. Absorbance vs. λ (nm).

3.3.3.2. Aminophospholipids, glucose, L-arginine and creatine

The sample containing only aminophospholipids (Control) showed a higher absorbance and number of peaks from 270 to 200 nm (Figure 5). When glucose, L-arginine or creatine was added the absorption spectra were similar to each other, however, the intersections that occur at 360, 305 and 270 nm between the three spectra suggest the formation of different compounds with L-arginine or creatine. A slight decrease in absorbance is observed with the addition of L-arginine to the mixture containing aminophospholipids with glucose, although

it does not appear to be significant, it suggests a protective effect of this amino acid on the glycation of aminophospholipids by glucose.

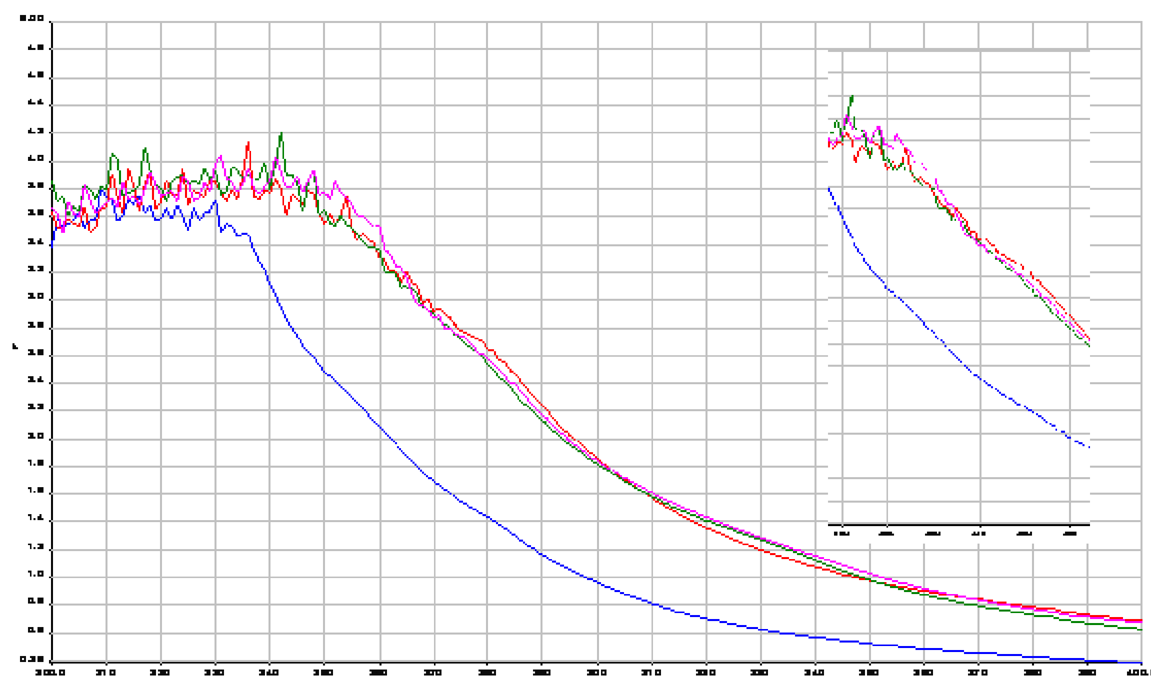

Figure 5: Aminophospholipids at day 61 of incubation with glucose, L-arginine and creatine. Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 2. Aminophospholipids + glucose. Green color: Vial 5. Aminophospholipids + glucose + L-arginine. Pink color: Vial 8. Aminophospholipids + glucose + creatine. Absorbance vs. λ (nm).

3.3.3.3. Aminophospholipids, β-hydroxybutyrate, L-arginine and creatine

The combination of aminophospholipids with β-hydroxybutyrate showed a greater number of peaks in the region of 290 to 200 nm (Figure 6). The addition of both L-arginine and creatine caused a decrease in the absorption spectrum, which suggests inhibition of the formation of reaction products. When the effect of L-arginine and creatine is compared, creatine appears to have a better protective effect since the absorbance pattern is smaller and several peaks in the range of 290 to 260 nm disappear. An intersection point between both spectra is also observed at 260 nm.

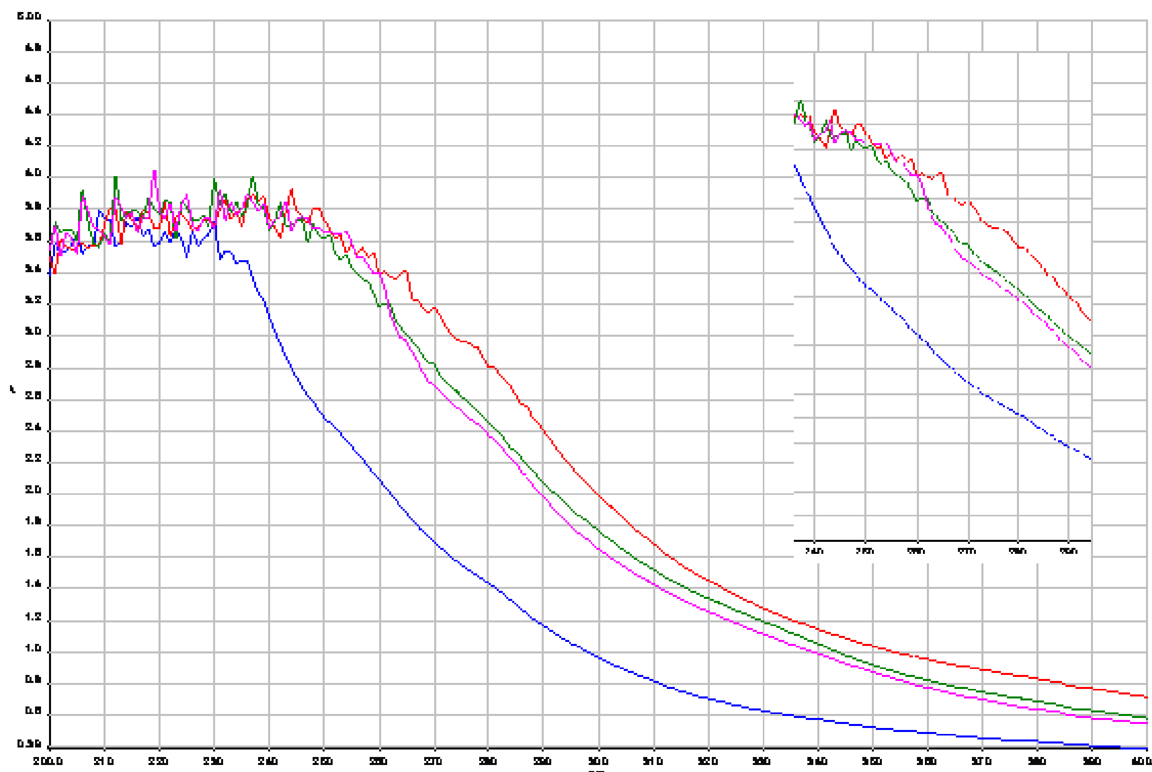

Figure 6: Aortic aminophospholipids at day 61 of incubation with β-hydroxybutyrate, L-arginine and creatine. Blue Color: Vial 1. Aminophospholipids (Control). Red color: Vial 3. Aminophospholipids + β-hydroxybutyrate. Green color: Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Pink color: Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Absorbance vs. λ (nm).

3.3.3.4. Aminophospholipids, acetoacetate, L-arginine and creatine

The combination of aminophospholipids with acetoacetate produced a lower absorbance pattern compared to the spectra obtained when these are combined with L-arginine or creatine. The control sample shows changes in the region of 290 to 240 nm with poorly defined peaks (Figure 7). The addition of L-arginine has an absorbance pattern similar to that of aminophospholipids with acetoacetate and with better defined peaks especially in the region of 280 to 200 nm. The addition of creatine to the mixture of aminophospholipids with acetoacetate produced a greater number of peaks practically from 300 to 210 nm. The absorbance recorded was greater. The difference in the formation of various products could be related to the chemical structure of acetoacetate.

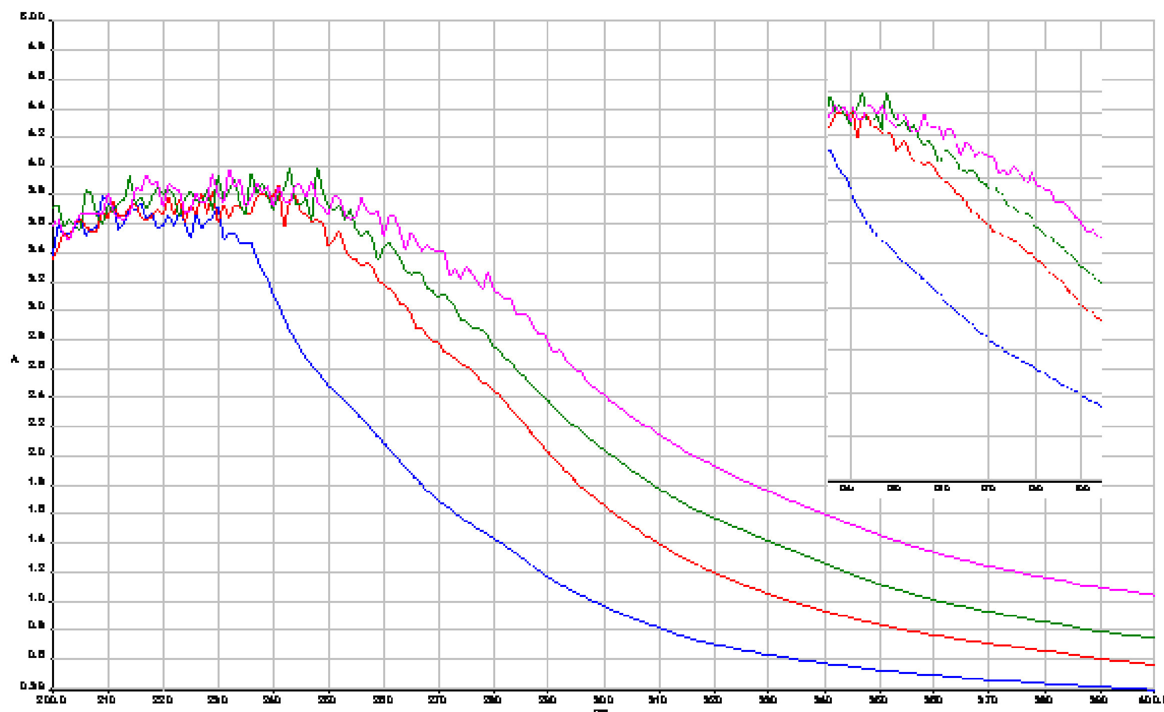

Figure 7: Aortic aminophospholipids at day 61 of incubation with acetoacetate, L-arginine and creatine. Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 4. Aminophospholipids + acetoacetate. Green color: Vial 7. Aminophospholipids + acetoacetate + L-arginine. Pink color: Vial 10. Aminophospholipids + acetoacetate + creatine. Absorbance vs. λ (nm).

3.3.3.5. Comparison of the effect of L-arginine in the reaction mixtures of aminophospholipids with glucose, β-hydroxybutyrate and acetoacetate

Figure 8 compares the effect of L-arginine in mixtures of aminophospholipids with glucose, β-hydroxybutyrate and acetoacetate. Systems containing glucose and β-hydroxybutyrate had similar absorbance patterns, slightly lower for β-hydroxybutyrate and with better defined peaks in the region of 280 to 220 nm for acetoacetate. These results suggest that L-arginine exerts a protective effect on the reaction with β-hydroxybutyrate.

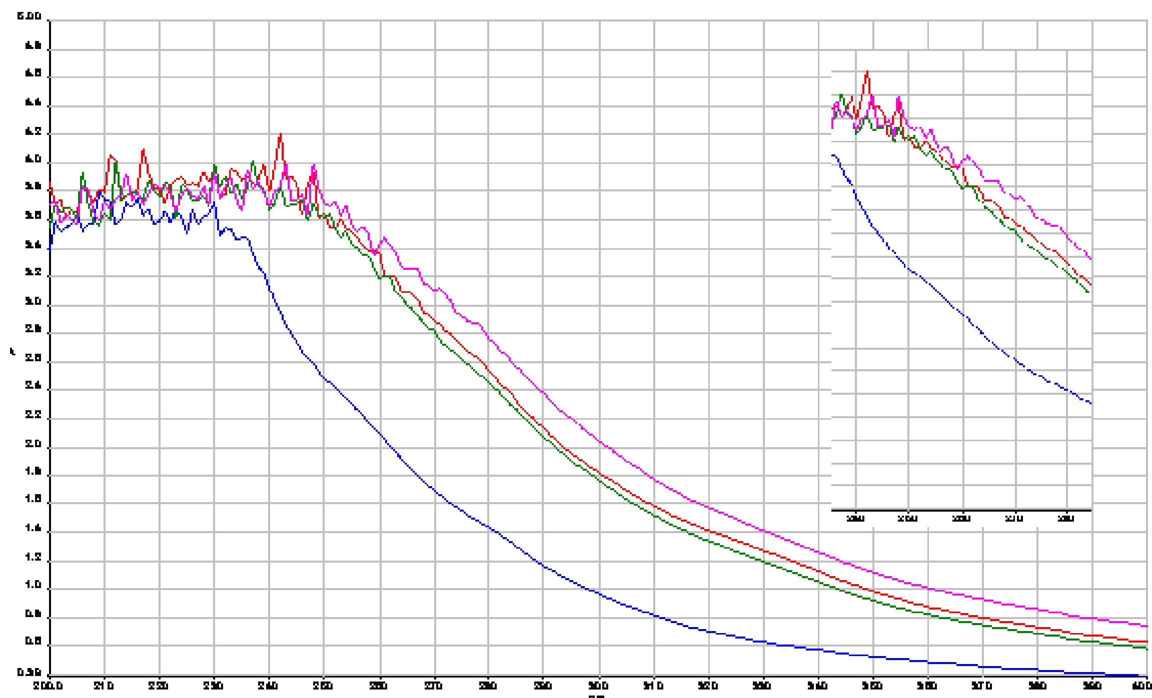

Figure 8: Aortic aminophospholipids at day 61 of incubation with L-arginine. Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 5. Aminophospholipids + glucose + L-arginine. Green color: Vial 6. Aminophospholipids + β-hydroxybutyrate + L-arginine. Pink color: Vial 7. Aminophospholipids + acetoacetate + L-arginine. Absorbance vs. λ (nm).

3.3.3.6. Comparison of the effect of creatine in the reaction mixtures of aminophospholipids with glucose, β-hydroxybutyrate and acetoacetate

The effect of creatine on the reaction of aminophospholipids with glucose, β-hydroxybutyrate and acetoacetate is compared in Figure 9. Although the spectra of glucose and β-hydroxybutyrate are similar, in the region of 290 to 220 nm there are differences in absorption patterns with an intersection point at 260 nm, being slightly higher in the presence of glucose. The absorbance is greater with acetoacetate defining a greater number of peaks from 300 to 200 nm. With creatine, the incubation system with β-hydroxybutyrate had lower absorption and number of peaks compared to systems containing glucose and acetoacetate, which suggests that creatine has a greater inhibitory effect on the reaction with β-hydroxybutyrate, taking into account that at lower absorption lower concentration of glycation products (Figure 9).

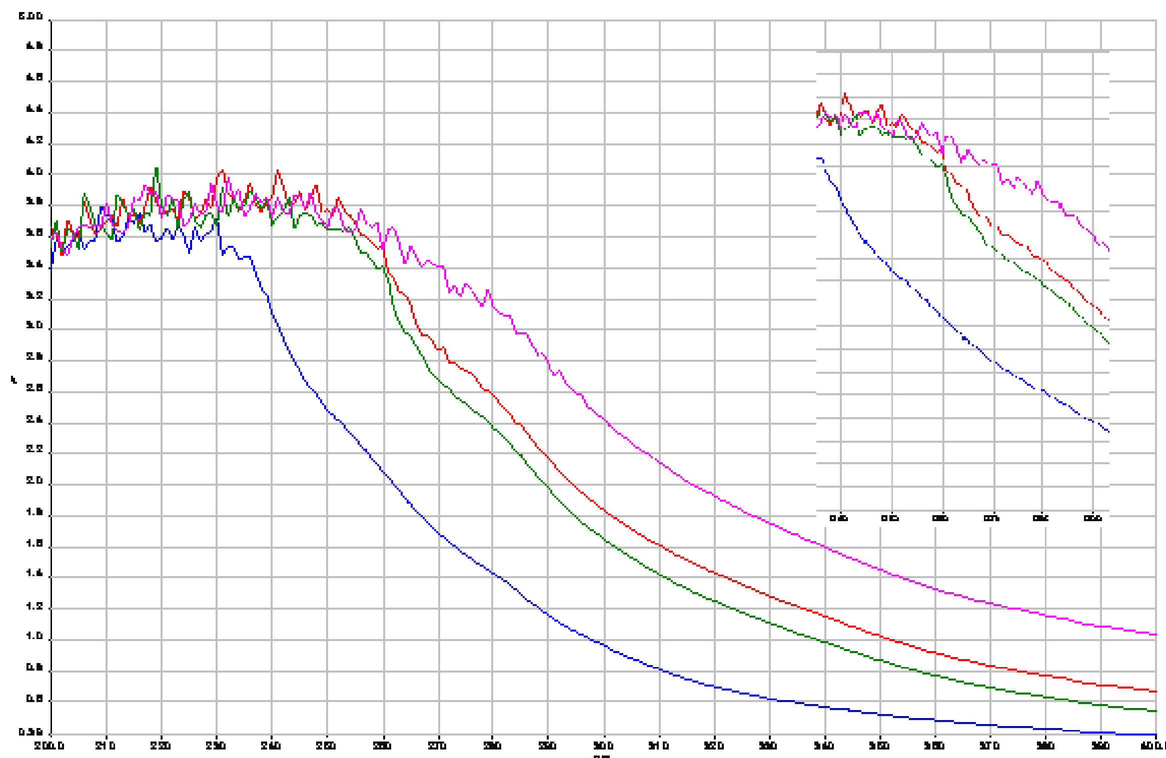

Figure 9: Aortic aminophospholipids at day 61 of incubation with creatine. Blue color: Vial 1. Aminophospholipids (Control). Red color: Vial 8. Aminophospholipids + glucose + creatine. Green color: Vial 9. Aminophospholipids + β-hydroxybutyrate + creatine. Pink color: Vial 10. Aminophospholipids + acetoacetate + creatine. Absorbance vs. λ (nm).

4. DISCUSSION

In humans acetyl-CoA formed in the liver during β-oxidation of fatty acids can enter the citric acid cycle or can be converted to β-hydroxybutyrate and acetoacetate by ketogenesis.[29] In healthy adults the liver can form up to 185 g of ketone body per day.[24] The process includes formation of acetoacetyl coenzyme A, conversion of acetoacetyl coenzyme A to β-hydroxy-β-methylglutaryl coenzyme A and then to acetoacetate; and finally reduction of acetoacetate to β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase.[22] β-Hydroxybutyrate and acetoacetate serve as metabolic fuels for peripheral tissues particularly skeletal muscle and heart. The brain, under normal circumstances, uses glucose as a source of energy, but when glucose is not available, ketone bodies become the brain's main source of fuel.[22] According to the proportion of circulating ketone bodies after eating food, the ratio of β-hydroxybutyrate / acetoacetate is approximately 1.0 but can be increased to 6 in prolonged fasting.[30] In some pathophysiological conditions such as diabetic ketoacidosis, circulating

levels of ketone bodies above 25 mM can be achieved, this occurs more frequently in type 1 diabetic patients[31] and less common in type 2 diabetics.

Untreated diabetes mellitus leads to overproduction of ketone bodies, with several associated medical problems. A single episode of moderate/severe diabetic ketoacidosis in young children at diagnosis has been associated with lower cognitive scores and altered brain growth.[32] The increased blood levels of β- hydroxybutyrate and acetoacetate lower the blood pH, causing the condition know as acidosis.[22] Extreme acidosis can lead to coma and in some cases death. Ketone bodies are also filtered in large quantities by the kidneys, and the fraction that is not re-absorbed is excreted in the urine. Ketone bodies in the blood and urine of untreated diabetes can reach extraordinary levels (< 3 mg/100 mL normal blood vs.90 mg/100 mL extreme ketosis (in untreated diabetes) and ≤125 mg/24 h vs 5 000 mg/24 h, a condition called ketosis.[22] We have observed that the ratio of glucose / ketone bodies in urine varies from 5.0 to 6.66 in type 2 diabetic patients with poor metabolic control.

In chronic diseases including diabetes mellitus protein, lipid or nucleic acid modifications by glycation are involved. The glycation of hemoglobin and cerebral aminophospholipids by ketone bodies in the absence of carbohydrates has been demonstrated. In an effort to attenuate glycation by ketone bodies, several substances with antiglycating properties have been tested. Among them, glycine, glycylglycine, aminoguanidine, L-arginine, polyamines and urea.[25,26]

Here, in the searching for new substances with the capacity to protect human arteries from toxic effects of ketone bodies, the *in vitro* effect of L-arginine and creatine on the glycation of aminophospholipids was studied. The aminophospholipids were combined with elevated concentrations of glucose and ketone bodies simulating hyperglycemia conditions and incubated for 61 days in the absence or in the presence of L-arginine or creatine. Interation of aminophospholipids, ketone bodies and antiglycant agents was analyzed by three ways.

4.1. Thin layer chromatography

The results of the chromatographic analysis show that using the same procedure that was followed in the isolation of aminophospholipids from erythrocyte membranes, aortic aminophospholipids were selectively isolated. As can be seen in Figure 1, these results reveal a greater diversity of aminophoslipids in the aorta. When the aminophospholipids were incubated for 61 days with glucose and ketone bodies with or without antiglicant substances, changes occurred not only in the intensity of the spots but also in the displacement expressed

in terms of R_f (Figure 2). Samples labeled A5, A6 and A7 correspond to aminophospholipids incubated with glucose, β-hydroxybutyrate and acetoacetate in the presence of L-arginine, respectively, compared to the control sample (A1, Vial 1). Samples A8, A9 and A10 correspond to aminophospholipids incubated with glucose, β-hydroxybutyrate and acetoacetate in the presence of creatine. The R_f values of the spots that showed greater intensity are presented in Table 1. In the case of samples A2, A3 and A4 the intensity of the spots is not very clear, suggesting that the aminophospholipids reacted with glucose and ketone bodies. When L-arginine or creatine was added, changes in R_f values and the appearance of compounds whose formation was dependent on incubation time were observed.

The behavior was similar to that observed for aminophospholipids isolated from red cell membrane. In the presence of L-arginine, greater intensity was observed in some components and variation in R_f values. On the other hand, seen by the R_f values from day 12 of incubation, creatine showed interaction when it was added to the systems containing aminophospholipids, glucose and ketone bodies (Figure 1, Table 1). In the case of the control sample, the spots were very thin which could be attributed to the degradation of lipids by oxidation, as observed for erythrocyte membrane aminophospholipids, occurred around day 12 of incubation. However, it is interesting to note that both L-arginine and creatine appear to exert a protective effect on the aminophospholipids since on day 61 of incubation intense stains suggesting the preservation of aminophospholipids are still observed, especially in the presence of L-arginine (Figure 1).

4.2. Fluorescence Analysis

The results of the fluorescence analysis of the different mixtures are summarized in Table 2. Similar pattern to that observed for isolated erythrocyte membrane aminophospholipids was obtained. The control sample seemed to degrade between days 12 and 19 showing an increase in fluorescence (Figure 3A). Both glucose and ketone bodies reacted with the aminophospholipids forming fluorescent compounds, which corroborates the results obtained by thin layer chromatography.

The comparative analysis in the emission range 355/460 nm also shows that the reaction with glucose occurred rapidly, while for β-hydroxybutyrate it was in the emission range of 320/460 nm. The single-factor ANOVA test confirmed that there is a significant difference in both cases ($P < 0.05$). With the Dunett test, a comparison was made between all incubation

and control mixtures. The result of this test demonstrates that in both cases L-arginine presented a significant difference (P <0.05), which is consistent with the graphic analysis of fluorescence means, thus demonstrating the inhibitory effect of L-arginine. Some significant differences are indicated in Table 2.

4.3. Ultraviolet spectroscopy

The reaction of glucose and ketone bodies with aortic aminophospholipids was also confirmed by ultraviolet spectroscopy. Through this technique it was possible to see that the compound that showed the highest reactivity was β-hydroxybutyrate, while fluorescence was glucose, although the difference is not significant. In the presence of glucose, L-arginine inhibits glycation more efficiently. The mixture of aminophospholipids and glucose showed lower reactivity than the creatine-containing system, which may be due to the fact that creatine can form alternate compounds with ketone bodies, suggesting this greater potential for inhibition. In the presence of β-hydroxybutyrate, creatine had a greater inhibitory effect.

When the aminophospholipids were only combined with acetoacetate, the reaction was lower compared to systems that also contained L-arginine or creatine. Mixtures containing L-arginine or creatine showed greater absorbance and a similar amount of peaks, suggesting that these substances react with the carbonyl group and favor the formation of several products. Creatine had a greater inhibitory effect compared to L-arginine.

5. COMMENTS

The participation of AGEs in the development of complications affecting macro and microvasculature is well documented.[33, 34] Although macrovascular disease is the major cause of mortality of all diabetic complications, vascular disease can manifest itself in the microvasculature leading to blindness, kidney disease, and various peripheral neuropathies.[35] Disease of the kidneys accounts for the highest rate of mortality of any microvascular complication. AGEs may activate the receptor for AGEs (RAGE), which is present on the surface of all cells relevant to atherosclerotic processes, triggering oxidative estress, inflammation and apoptosis.[36] Studies in rodent models of macro- and microvascular disease have demonstrated that blockade of RAGE can prevent development of disease. These observations highlight RAGE as a therapeutic target for treatment of diabetic vascular disease.[34]

Most of the knowledge about AGEs and their relationship with the development of chronic diseases have been obtained from the study of carbohydrate reactions, particularly glucose with biological molecules containing primary amino groups[37], as well as the reactions of the products of its autoxidation; glyoxal and methylglyoxal.[38] However, due to the heterogeneous nature of the adducts, the stability during isolation and the variation in the environmental conditions of the reactions, the elucidation of the AGE structures of how they are formed *in vivo* are not fully known.[39] Some AGEs result from the reaction of glucose with proteins such as 2-(2-furoyl)-4(5)-furanyl-1H-imidazole, 1-alkyl-2-formyl-3,4-glycosyl-pyrrole, and pyrraline.[39,40] have been identified and used as a model for the investigation of crosslinking mechanisms.

On the other hand, lipid peroxidation is another important source of carbonyl intermediates that contribute to the formation of AGEs and consequently to the development of vascular diseases. Several aldehydes are produced by the peroxidation of polyunsaturated fatty acids including malondialdehyde. Malonaldehyde is produced mainly from peroxidation of polyunsaturated fatty acids with more than 3 methylene-interrupted double bonds. Thus, it arises mainly from arachidonic acid and docosanoic acid.[41, 42] The malonaldehyde levels in normal human urine has been reported to be in the range of 0.2-0.8 μM.[42] Other aldehydes, hydroxyalkenales; 4-hydroxy-2(E)-hexenal, 4-hydroxy-2 (E)-nonenal, and 4-hydroxy-dodecadienal, also derived from the oxidation of polyunsaturated fatty acids react with molecules containing primary amino groups such as aminophospholipids.[43] Adduct formation between aminophospholipids and hydroxyalkenals has been described in human blood platelets in response to oxidative stress and in retinas of streptozotocin-induced diabetic rats.[44] These adducts have been characterized and evaluated and it has been suggested that they could be used as specific markers of membrane disorders occurring in pathophysiological states with associated oxidative stress and might affect cell function.[44] Other studies on glycated phosphatidylethanolamines containing unsaturated fatty acids; 18:1, 18:2 and 18:4 after ultraviolet photo-irradiation showed mainly hydroperoxy derivates due to oxidation of unsaturated fatty acyl chains. In addition to this, several new photooxidation products formed due to oxidative cleavages of the glucose moiety, namely between C1 and C2, C2 and C3 and C5 and C6 of this sugar unit, have been asssociated in the etiology of diabetic retinopathy and in diabetic retinal microvascular complications.[45]

6. CONCLUSION

In conclusion, these results provide evidence on the diversity of aminophospholipids of the human aorta. The glycation of these aminophospholipids by the ketone bodies; β-hydroxybutyrate and acetoacetate has been confirmed by three technical procedures; thin layer chromatography, fluorescence and ultraviolet spectroscopy. There is sufficient evidence of the relationship of AGEs with the development of cardiovascular disease. Several substances have been tested to inhibit glycation. The beneficial effect of L-arginine and polyamines among others has been reported.[46] Here, the effect of L-arginine and its metabolite, creatine, has been demonstrated. Both molecules are formed in the body, while arginine is required in multiple metabolic fates, creatine, is converted to phosphocreatine, a molecule that stores energy for skeletal muscle and brain. Creatine glycation in hyperglycemia could result in phosphocreatine deficiency. Since the main consequence of long-term hyperglycemia is the increased incidence of disease of the vasculature, this could be prevented with the administration of L-arginine and creatine in addition to good metabolic control.

7. ACKNOWLEDGEMENTS

This work was presented at the 3rd International Congress & Expo on Heart & Cardiology. September 19-20, 2019. Miami, USA.

The authors thanks to Paulina Hernández Sánchez López, B. Pharm. for her technical assistance.

8. REFERENCES

1. Westwood ME, Thornalley PJ. Glycation and advance glycation end products. In: The Glycation Hypothesis of Atherosclerosis, Colaco C. (Ed). Landes Bioscience and Chapman & Hall. Austin, Texas, U.S.A. 1997. Chapter 3. pp.57-87.
2. IUPAC Commission on the nomenclature of organic chemistry (CCOC), IUPAC-IUB Commission on Biochemical Nomenclature (CBN). Tentative rules for carbohydrate nomenclature. Part I, 1969. Biochem J., 1971; 125: 673-695.
3. Vlassara H. Recent progress in advanced glycation end products and diabetic complications. Diabetes., 1997; 46(Suppl.2): S19–S25.
4. Vlassara H, Palace MR. Glycoxidation: the menace of diabetes and aging. Mt Sinai J Med., 2003; 70: 232–241.

5. O´Brien J. Introduction to the Maillard reaction. In: The Glycation Hypothesis of Atherosclerosis, Colaco C. (Ed). Landes Bioscience and Chapman & Hall. Austin, Texas, U.S.A. 1997. Chapter 2. pp.29-55.
6. Thornalley PJ, Lanborg A, Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of protein by glucose. Biochem J., 1999; 344: 109-116.
7. Signh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. Diabetología., 2001; 44: 129-146.
8. Knecht KJ, Feather MS, Baynes JW. Detection of 3-deoxyfructose and 3-deoxyglucosone in human urine and plasma: evidence for intermediate stages of the Maillard reaction *in vivo*. Arch Biochem Biophys., 1992; 294: 130-137.
9. Chappey O, Dosquet C, Wautier MP, Wautier JL. Advanced glycation end products, oxidant stress and vascular lesions. Eur J Clin Invest., 1997; 27: 97-108.
10. Ahmed N. Advanced glycation end products–role in pathology of diabetic complications. Diabetes Res Clin Pract., 2005; 67: 3–21.
11. Bunn HF, Gabbay KH, Gallop PM. The glycosylation of hemoglobin: relevance to diabetes mellitus. Science., 1978; 200: 21-27.
12. Sheyu L, Donnelly L, Hapca S, Zhou K, Pearson ER. Visit-to-visit HbA1c variability is associated with cardiovascular disease and microvascular complications in patients with newly diagnosed Type 2 diabetes. Diabetes Care., 2019; dc190823.
13. Glenn JV, Stitt AW, The role of advanced glycation end products in retinal ageing and disease. Biochim Biophys Acta., 2009; 1790: 1109-1116.
14. Fu MX, Requena JR, Jenkins AJ, Lyons TJ, Baynes JW, Thorpe SR. The advanced glycation end product, N Epsilon-(carboxymethyl) lysine, is a product of both lipid peroxidation and glycoxidation reactions. J Biol Chem., 1996; 271(17): 9982-9986.
15. Uchida K, Kanematsu M, Morimitsu Y, Osawa T, Noguchi N, Niki E. Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and its conjugate with lysine residues in oxidized low density lipoproteins. J Biol Chem., 1998; 273(26): 16058-16066.
16. Park MH, Igarashi K. Polyamines and their metabolites as diagnostic markers of human diseases. Biomol Ther (Seoul)., 2013; 21(1): 1–9.
17. Tomitori H, Usui T, Saeki N, Ueda S, Kase H, Nishimura K, Kashiwagi K, Igarashi K. Polyamine oxidase and acrolein as novel biochemical markers for diagnosis of cerebral stroke. Stroke., 2005; 36: 2609-2613.

18. Taneda S, Monnier VM. ELISA of pentosidine, an advanced glycation end product, in biological specimens. Clin Chem., 1994; 40: 1766-1773.
19. Onorato JM, Thorpe SR, Baynes JW. Immunohistochemical and ELISA assays for biomarkers of oxidative stress in aging and disease. Ann. N.Y. Acad. Sci., 1998; 854: 277–290.
20. Hartog JW, Voors AA, Bakker SJ, Smit AJ, Van Veldhuisen DJ. Advanced glycation end-products (AGEs) and heart failure: pathophysiology and clinical implications. EJHF., 2007; 9: 1146–1155.
21. Hegab Z, Gibbons S, Neyses L, Mamas MA. Role of advanced glycation end products in cardiovascular disease. WJC., 2012; 4: 90–102.
22. Nelson, D.L, Cox, M.M. Lehninger Principles of Biochemistry. Worth Publishers. 3rd ed. New York, USA. 2000. pp. 615-618.
23. Davidson, JK. Diabetic ketoacidosis and the hyperglycemic hyperosmolar state. In: Clinical Diabetes Mellitus. A Problem-Oriented Approach. Third Edition. Davidson, J. K. (Ed). Thieme. New York, USA. 2000. pp. 479-498.
24. Laffel, L. Ketone bodies: a review of physiology, pathophysiology and application of monitoring to diabetes. Diabetes Metab Res Rev., 1999; 15: 412-426.
25. Xie J, García-Pérez E, Méndez JD. *In vitro* glycation of hemoglobin by acetone and β-hydroxibutirate. World Appl Sci J., 2007; 2(2): 99-106.
26. Aguilar-Hernández M, Méndez JD. *In vitro* glycation of brain aminophospholipids by acetoacetate and its inhibition by urea. Biomed Pharmacother., 2007; 61(10): 693-697.
27. Méndez JD. Experimentos Básicos de Bioquímica. Ed. Prado. México, D. F. 2001; pp. 89-96.
28. Ratkowski DA, Evans MA, Alldredge JR. Cross-over experiments. Design, analysis and application. Statistics Textbooks and Monographs, 135, Marcel Dekker, New York, USA. 1993.
29. Voet D, Voet JG. Biochemistry. 2nd Ed. John Wiley & Sons, Inc. New York, USA. 1995. pp. 678 - 680.
30. Mitchell GA, Kassovska-Bratinova S, BoukaftaneY, Robert MF, Wang SP, Ashmarina L, Lambert M, Lapierre P, Potier E. Medical aspects of ketone body metabolism. Clin Invest Med., 1995; 18: 193-216.
31. Bonnefont JP, Specola NB, Vassault A, Lombes A, Ogier H, de Klerk JB, Munnich A, Coude M, Paturneau-Jouas M, Saudubray JM. The fasting test in paediatrics: application

to the diagnosis of pathological hypo-and hyperketotic states. Eur J Pediatr., 1990; 150: 80-85.

32. Aye T, Mazaika PK, Mauras N, Marzelli MJ, Shen H, Hershey T, Cato A, Weinzimer SA, White NH, Tsalikian E, Jo B, Reiss AL; Diabetes Research in Children Network (DirecNet) Study Group. Impact of early diabetic ketoacidosis on the developing brain. Diabetes Care., 2019; 42(3): 443-449.
33. Stitt, AW, Bucala R, Vlassara H. Atherogenesis and advanced glycation: Promotion, progression and prevention. Ann NY Acad Sci., 1997; 811: 115-129.
34. Hudson BI, Wendt T, Bucciarelli LG, Rong LL, Naka Y, Yan SF, Schmidt AM. Diabetic vascular disease: it's all the RAGE. Antioxid Redox Signal. 2005; 7(11-12): 1588-1600.
35. Nathan DM. Long-term complications of diabetes mellitus. N Engl J Med., 1993; 328: 1676 –1685.
36. Stirban A, Gawlowski T, Roden M. Vascular effects of advanced glycation end products: Clinical effects and molecular mechanisms. Mol Metab., 2014; 3: 94 –108.
37. Méndez JD, Xie J, Aguilar-Hernández M, Méndez-Valenzuela V. Molecular susceptibility to glycation and its implication in diabetes mellitus and related diseases. Mol Cell Biochem., 2010; 344(1-2): 185-193.
38. Chetyrkin S, Mathis M, Pedchenko V, Sanchez OA, Hayes McDonald W, Hachey DL, Madu H, Stec D, Hudson B, Voziyan P. Glucose autoxidation induces functional damage to proteins via modification of critical arginine residues. Biochemistry., 2011; 50(27): 6102-6112.
39. Pongor S, Urlich PC, Bencsath FA, Cherami A. Aging of proteins: isolation and identification of a fluorescent chromophore from the reaction of polypeptides with glucose. Proc Nat Acad Sci. USA., 1989; 2684-2688.
40. Sell D, Monnier VM. Structure elucidation of a senescence cros-slink from human extracellular matrix. J Biol Chem., 1984; 264: 21597-21602.
41. Pryor AW, Stanley PA. A suggested mechanism for the production of malonaldehyde during the autoxidation of polyunsaturated fatty acids. Nonenzymatic production of prostaglandin endoperoxides during autoxidation. J Org Chem., 1975; 40: 3615-3617.
42. Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med., 1991; 11: 81-128.
43. Bacot S, Bernoud-Hubac N, Chantegrel B, Deshayes C, Doutheau A, Ponsin G, Lagarde M, Guichardant M. Evidence for *in situ* ethanolamine phospholipid adducts with hydroxy-alkenals. J Lipid Res., 2007; 48(4): 816-825.

44. Guichardant M, Bacot S, Molière P, Lagarde M. Hydroxy-alkenals from the peroxidation of n-3 and n-6 fatty acids and urinary metabolites. Prostaglandins Leukot Essent Fatty Acids., 2006; 75(3): 179-182.
45. Melo T, Silva EM, Simões C, Domingues P, Domingues MR. Photooxidation of glycated and non-glycated phosphatidylethanolamines monitored by mass spectrometry. J Mass Spectrom., 2013; 48(1): 68-78.
46. Méndez, J. D.: New perspectives on the research and treatment of type 2 diabetes mellitus and its complications. The role of L-arginine and polyamines. WJPR., 2019; 8(4): 238-244.