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ACETONE, THE THIRD KETONE BODY. II. ACTIONS ON ERYTHROCYTE MEMBRANE AND HEMOGLOBIN IN TOTAL HUMAN BLOOD

José D. Méndez* and Edith García-Pérez

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

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***Corresponding Author**
Dr. José D. Méndez
Medical Research Unit in Metabolic Diseases, Cardiology Hospital. National Medical Center, Instituto Mexicano del Seguro Social, 06703 Mexico City, Mexico.

ABSTRACT

Previously, was informed by our group that glycated hemoglobin was produced in hemoglobin samples incubated with acetone and β-hydroxybutyrate alone, suggesting that glycated hemoglobin can be non-enzymatically formed *in vitro* from ketone bodies in the absence of carbohydrates, providing evidence that ketone bodies contribute to the development of complications of un-controlled type 2 diabetes and mainly of type 1 diabetes. In this study, the effect of acetoacetate and acetone at concentrations similar to those found in ketoacidosis was studied in total human blood. Blood samples (3 mL) of 48 volunteers were grouped and treated as follow: 1. D-glucose (Control), 2. Acetoacete, 3. D- glucose plus acetoacetate, 4. Acetone. In order to inhibit glycation, glycine, glycilgycine, urea, aminoguanidine and L-arginine were added. Samples were made in duplicate with their respective controls and incubated in the dark for 90 days at 37º C. Concentrations of hemoglobin and glycated hemoglobin were measured at the start and at the end of the study. Glycated hemoglobin was determined by turbidimetric immunoassay using specific antibodies and by fluorescence testing using two pairs of filters: Excitation 320/emission 460 nm and excitation 355 / emission 460 nm. High concentrations of glucose produced glycation of hemoglobin in intact erythrocytes. Acetoacete did not show relevant glycation of hemoglobin in the conditions of this study. When acetone was added to the blood samples, an inmediate denaturing effect on plasma proteins was observed and erythrocyte membrane disaggregation occurred. Glycation by glucose was inhibited by L-arginine while no inhibitory effect on hemoglobin glycation by glycine, glycylglycine, aminoguanidine and

urea was observed. In conclusion, as expected major glycation of hemoglobin was observed when total blood was exposed to high concentration of glucose. No relevant glycation was observed with acetoacetate. Disaggregation of the membrane erythrocyte and denaturing effect on proteins was caused by acetone. Additional studies are necessary to evaluate the effect of different concentrations of acetone on the integrity of the erythrocyte membrane, as well as other blood cells and subcellular organelles, and on plasma proteins in short periods of time.

KEYWORDS: Human Erythrocyte, Diabetes Mellitus, Ketone Bodies, Acetoacetate, Acetone, Advanced Glycation end Products, L-arginine, Aminoguanidine, L-glycine, Glycilglycine, Urea.

1. INTRODUCTION

Three molecules are known as ketone bodies; the first; acetoacetic acid, the second, β-hydroxybutyrate, and the third, acetone.[1] Acetoacetate (ionized form of acetoacetic acid) accumulates during fatty acid metabolism under low carbohydrate conditions, while β-hydroxybutyrate is formed from reduction of acetoacetate in a reaction catalyzed by β-hydroxybutyrate dehydrogenase in the mitochondria.[2] Acetone is generated either by spontaneous decarboxylation of acetoacetate or decarboxylation catalyzed by acetoacetate decarboxylase.[1, 3] Acetoacetate and β -hydroxybutyrate provide cells with acetyl-CoA that is subsequently oxidized for energy that the body requires, via the citric acid cycle.[1]

In healthy people, acetone is formed in very small amounts, it is described as a biologically inert side product.[4] Its levels vary depending on many factors, such as infancy, pregnancy, lactation, physical exercise, dieting and starvation.[5] It has been described that these amounts of acetone usually do not cause health problems. However, 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.[6]

In severe ketoacidosis (plasma glucose (mg/dL): >250), arterial pH: <7.00, serum bicarbonate: <10 mEq/L),[7] the increased blood levels of β-hydroxybutyrate and acetoacetate lower the blood pH.[2] Ketone bodies have been shown to affect vascular integrity and permeability and contribute to edema formation.[7] Extreme acidosis can lead to coma and in some cases death.[8, 9] In the blood and urine of untreated diabetic patients can reach

extraordinary levels (< 3 mg/100 mL normal blood vs.90 mg/100 mL extreme ketosis and ≤125 mg/24 h vs 5 000 mg/24 h.[2,10] The ratio of glucose / ketone bodies in urine can vary from 5.0 to 6.66 in type 2 diabetics with poor metabolic control. Acetone, as volatile substance is excreted, mostly by the kidneys and lungs (breath), but also is metabolized to pyruvate and glucose[11] leading to hyperglycemia, or converted to isopropyl alcohol by alcohol dehydrogenase. Isopropyl alcohol can be converted to methylglyoxal, a precursor in the formation of advanced glycation end products which is associated to the development of chronic diabetic complications.[12, 13] Plasma-acetone remained elevated for periods of up to 42 hours, long after blood-glucose, acetoacetate, and β-hydroxybutyrate levels had returned to normal. Previously, we informed on the glycation of hemoglobin directly caused by β-hydroxybutyrate and acetone.[14] Interestingly, in samples without reducing sugars moderate levels of glycated hemoglobin were detected by turbidimetric inhibition immunoassay using specific HbA1c antibodies. The study of fluorescent intensity provided evidence that more fluorophores were yielded in hemoglobin samples incubated with both glucose and acetone than glucose or acetone alone, meaning that in addition to the formation of glycated hemoglobin, other fluorescent compounds were produced during 90 days of incubation which could be associated with glucose autoxidation products, however, it was not studied. Among eight inhibitors used in that study, major inhibition was caused by urea. Urea is formed from L-arginine, an excellent regulator of hyperglycemia,[15] while urea seems to be a natural regulator of glycation.[16]

This study was designed to evaluate the role of acetoacetate and acetone in the *in vitro* glycation of hemoglobin, using human erythrocyte as an experimental model. Additionally the effect of several substances with possible antiglycant effect was tested.

2. MATERIALS AND METHODS

2.1. Reagents for glycated hemoglobin determination:

A Roche Tina-quant® Hemoglobin A1c II brand immunoassay kit was used to determine the concentration of glycated hemoglobin (HbA1c) during this study. The kit consists of the following reagents:

Reagent 1. 0.025 M (2- (N-Morpholino) ethane sulfonic acid (MES) buffer; 0.015 M Tris(hydroxymethyl)aminomethane (TRIS) buffer, pH 6.2; HbA1c antibodies (sheep serum): ≥ 0.5 mg / mL; stabilizers.

Reagent 2. 0.025 M MES buffer solution; (TRIS) buffer, pH 6.2; polyhapten HbA1c: ≥ 8 µg / mL; stabilizers.

Reagent 3. 3a-d Hemolysate derived from human blood and sheep blood; Tetradecyl trimethyl ammonium bromide (Sigma-Aldrich): 9 g / L; stabilizers.

Reagent 4. Hemoglobin: 0.02 M phosphate buffer, pH 7.4; stabilizers.

2.2. D-glucose, Lithium acetoacetate and acetone (Sigma-Aldrich) were used to induce the advanced glycation end products formation.

2.3. L-glycine, aminoguanidine, urea and L-arginine (Merck), glycylglycine (Nutritional Biochemicals Corporation, Cleveland, OH, USA) were used to inhibit the advanced glycation end products formation.

2.4. Participants

Forty-eight volunteers, men and women, with the following characteristics were included in this study: 1. Age; between 18 and 65 years. 2. Weight; greater than 50 Kg. 3. Not pregnant or breastfeeding. 4. Without having suffered from hepatitis. 5. They did not present fever and any type of illness seven days prior to taking the sample. 6. No drug administration.

2.5. Blood samples collection

Blood samples (3.5 - 7.0 mL) were collected the day before the study and kept refrigerated.

2.6. Basal hemoglobin and glycated hemoglobin determination

Hemoglobin and glycated hemoglobin concentrations were determined before the start the experimental glycation process (Table 1). To calculate the concentration of HbA1c, the following formula was used, which is described in the kit manual:

$$\% \text{ HbA1c} = \left(87.6 \times \frac{\text{HbAc1 [g/dL]}}{\text{Hb [g/dL]}}\right) + 2.27$$

2.7. Basal fluorescence

The basal fluorescence of each sample was performed in a fluorometer in the wavelength ranges of 320/460 nm and 355 / 460 nm excitation / emission.

2.8. Glycation induction

Samples were grouped as follow: 1. D-glucose, 2. Acetoacetic acid, 3. D- glucose plus acetoacetic acid, 4. Acetone.

The substances used as inhibitors of hemoglobin glycation were added to each group: L-glycine, glycylglycine, urea, aminoguanidine and L-arginine, together with the respective controls. In order to make the experiment uniform, 3 mL of each blood sample were taken for the study. Each sample was made in duplicate. Table 2 shows the sample number with the respective inhibitor used.

Table 1: Hemoglobin and basal glycated hemoglobin concentration in healthy volunteers.

Volunteer Number	Hb (mg/dL)	HbA1c (mg/dL)	HbA1c %	Volunteer Number	Hb (mg/dL)	HbA1c (mg/dL)	HbA1c %
1	16.9	0.56	5.2	25	14.0	0.50	5.4
2	17.2	0.65	5.6	26	16.0	0.64	5.8
3	14.8	0.53	5.4	27	13.2	0.44	5.2
4	15.7	0.63	5.8	28	14.6	0.56	5.6
5	15.7	0.59	5.6	29	15.6	0.64	5.9
6	15.6	0.63	5.8	30	12.6	0.44	5.3
7	16.8	0.65	5.7	31	16.0	0.54	5.2
8	15.3	0.62	5.8	32	15.4	0.58	5.6
9	15.7	0.55	5.3	33	14.8	0.48	5.1
10	15.4	0.55	5.4	34	14.3	0.51	5.4
11	18.0	0.58	5.1	35	16.2	0.58	5.4
12	16.3	0.55	5.2	36	15.6	0.48	5.0
13	17.4	0.58	5.2	37	14.7	0.58	5.7
14	13.8	0.48	5.3	38	15.4	0.57	5.5
15	15.9	0.60	5.6	39	13.5	0.51	5.6
16	16.3	0.57	5.3	40	14.5	0.48	5.2
17	16.1	0.55	5.3	41	15.0	0.58	5.7
18	15.1	0.52	5.3	42	17.3	0.59	5.3
19	15.6	0.72	6.3	43	16.6	0.57	5.3
20	17.2	0.62	5.4	44	16.7	0.62	5.5
21	16.1	0.52	5.1	45	13.4	0.52	5.7
22	17.1	0.58	5.2	46	15.2	0.56	5.5
23	15.1	0.59	5.7	47	14.3	0.61	6.0
24	16.6	0.56	5.2	48	15.2	0.55	5.4

In the human body, under physiological conditions the hemoglobin concentration varies from 12.7-16.5 g / dL, on average the hemoglobin concentration is 14.5 g / dL. On the other hand, blood glucose values greater than 150 mg / dL are considered risk in the patient, or greater than 32 mM. In this study, a concentration of 35 mM glucose was used to induce glycation *in vitro*.[17]

The concentration of ketone bodies was 1.5 mg mL^{-1} and a concentration of 20 mM of inhibitors such as L-glycine, glycylglycine, urea, aminoguanidine and L-arginine.[18]

Table 2: Processing of blood samples for the induction of hemoglobin glycation and its prevention by different substances with inhibitory action.

Volunteer Number	Protein	Carbohydrate	Ketone Body	Inhibitor
1	Hemoglobin	D-glucose*		-
2	Hemoglobin	D-glucose*		-
3	Hemoglobin	D-glucose		L-Glycine
4	Hemoglobin	D-glucose		L-Glycine
5	Hemoglobin	D-glucose		Glycylglycine
6	Hemoglobin	D-glucose		Glycylglycine
7	Hemoglobin	D-glucose		Urea
8	Hemoglobin	D-glucose		Urea
9	Hemoglobin	D-glucose		Aminoguanidine
10	Hemoglobin	D-glucose		Aminoguanidine
11	Hemoglobin	D-glucose		L-Arginine
12	Hemoglobin	D-glucose		L-Arginine
13	Hemoglobin		Acetoacete*	-
14	Hemoglobin		Acetoacete*	-
15	Hemoglobin		Acetoacete	L-Glycine
16	Hemoglobin		Acetoacete	L-Glycine
17	Hemoglobin		Acetoacete	Glycylglycine
18	Hemoglobin		Acetoacete	Glycylglycine
19	Hemoglobin		Acetoacete	Urea
20	Hemoglobin		Acetoacete	Urea
21	Hemoglobin		Acetoacete	Aminoguanidine
22	Hemoglobin		Acetoacete	Aminoguanidine
23	Hemoglobin		Acetoacete	L-Arginine
24	Hemoglobin		Acetoacete	L-Arginine
25	Hemoglobin	D-glucose*	Acetoacete	-
26	Hemoglobin	D-glucose*	Acetoacete	-
27	Hemoglobin	D-glucose	Acetoacete	L-Glycine
28	Hemoglobin	D-glucose	Acetoacete	L-Glycine
29	Hemoglobin	D-glucose	Acetoacete	Glycylglycine
30	Hemoglobin	D-glucose	Acetoacete	Glycylglycine
31	Hemoglobin	D-glucose	Acetoacete	Urea
32	Hemoglobin	D-glucose	Acetoacete	Urea
33	Hemoglobin	D-glucose	Acetoacete	Aminoguanidine
34	Hemoglobin	D-glucose	Acetoacete	Aminoguanidine
35	Hemoglobin	D-glucose	Acetoacete	L-Arginine
36	Hemoglobin	D-glucose	Acetoacete	L-Arginine
37	Hemoglobin		Acetone*	-
38	Hemoglobin		Acetone*	-
39	Hemoglobin		Acetone	L-Glycine
40	Hemoglobin		Acetone	L-Glycine
41	Hemoglobin		Acetone	Glycylglycine
42	Hemoglobin		Acetone	Glycylglicine
43	Hemoglobin		Acetone	Urea
44	Hemoglobin		Acetone	Urea

45	Hemoglobin		Acetone	Aminoguanidine
46	Hemoglobin		Acetone	Aminoguanidine
47	Hemoglobin		Acetone	L-Arginine
48	Hemoglobin		Acetone	L-Arginine

* Control samples without inhibitor.

Each sample was labeled for identification, covered and handled according to the order shown in Table 2. The samples were properly processed to avoid any microbiological contamination. Then, the samples were incubated at 37º C, for 90 days.

2.9. Final glycated hemoglobin determination and fluorescence measurement.

At the end of the incubation time, each sample was shaken and read to analyze the amount of HbA1c using the Tina-quant® Hemoglobin A1c II kit from Roche. Subsequently, a 1: 5 dilution of each sample was made using tetradecyl trimethyl ammonium as diluent.

The fluorescence reading was performed as described previously for basal conditions.

3. RESULTS

As shown in Table 1, normal HbA1c concentrations are between 4-6%. The results of the determination of both hemoglobin and glycated hemoglobin obtained after 90 days of incubation at 37 ºC, are shown in Table 3.

Table 3: Hemoglobin and glycated hemoglobin concentration after 90 days of incubation.

Volunteer Number	Hb (mg/dL)	HbA1c (mg/dL)	HbA1c %	Volunteer Number	Hb (mg/dL)	HbA1c (mg/dL)	HbA1c %
1	17.20	0.99	7.3	25	15.60	0.79	6.7
2	19.10	1.01	6.9	26	17.60	0.79	6.2
3	18.60	0.95	6.7	27	17.00	0.86	6.7
4	20.40	0.92	6.1	28	19.40	0.98	6.7
5	19.10	1.26	8.0	29	17.80	0.98	7.1
6	17.20	1.09	7.8	30	14.00	0.74	6.9
7	17.90	1.08	7.6	31	21.00	1.01	6.5
8	19.90	1.32	7.9	32	17.30	0.77	6.2
9	18.60	1.44	9.0	33	18.60	1.24	7.9
10	17.90	1.23	8.2	34	19.40	1.30	8.1
11	21.30	0.60	4.7	35	18.60	0.63	5.2
12	18.80	0.00	2.3	36	20.40	0.60	4.8
13	18.50	0.00	2.3	37	ND	ND	ND
14	17.50	0.00	2.3	38	18.90	0.00	2.3
15	9.40	0.00	2.3	39	19.20	0.00	2.3
16	17.30	0.00	2.3	40	ND	ND	ND

17	17.90	0.00	2.3	41	ND	ND	ND
18	17.10	0.00	2.3	42	ND	ND	ND
19	19.60	0.00	2.3	43	ND	ND	ND
20	19.30	0.17	3.0	44	ND	ND	ND
21	19.90	0.08	2.6	45	49.40	0.11	2.5
22	19.90	0.20	3.1	46	33.30	0.23	2.8
23	14.90	0.15	3.2	47	34.60	0.19	2.7
24	22.00	0.20	3.1	48	20.40	0.16	2.9

ND= NOT DETERMINED.

In the case of samples 37, 38, 39, 40, 41, 42, 43, and 44, the HbA1c concentration could not be determined since they had the characteristic of being very viscous after incubation. These samples contained acetone as a glycation substance. It samples appear labeled as ND, which were not read.

The results obtained before the % HbA1c (1) incubation were compared with those obtained after the % HbA1c (2) incubation, as well as the relationship between them, which are shown in Table 4.

Table 4: Relationship between initial % HbA1c and final % HbA1c.

Sample Number	% HbA_{1c} initial (1)	% HbA_{1c} final (2)	% HbA_{1c} (2)/ % HbA_{1c} (1)	Sample Number	% HbA_{1c} initial (1)	% HbA_{1c} final (2)	% HbA_{1c} (2)/ % HbA_{1c} (1)
1	5.2	7.3	1.4038	25	5.4	6.7	1.2407
2	5.6	6.9	1.2321	26	5.8	6.2	1.0690
3	5.4	6.7	1.2407	27	5.2	6.7	1.2885
4	5.8	6.1	1.0517	28	5.6	6.7	1.1964
5	5.6	8.0	1.4286	29	5.9	7.1	1.2034
6	5.8	7.8	1.3448	30	5.3	6.9	1.3019
7	5.7	7.6	1.3333	31	5.2	6.5	1.2500
8	5.8	7.9	1.3621	32	5.6	6.2	1.1071
9	5.3	9.0	1.6981	33	5.1	7.9	1.5490
10	5.4	8.2	1.5185	34	5.4	8.1	1.5000
11	5.1	4.7	0.9216	35	5.4	5.2	0.9630
12	5.2	2.3	0.4423	36	5.0	4.8	0.9600
13	5.2	2.3	0.4423	37	5.7	ND	ND
14	5.3	2.3	0.4340	38	5.5	2.3	0.4182
15	5.6	2.3	0.4107	39	5.6	2.3	0.4107
16	5.3	2.3	0.4340	40	5.2	ND	ND
17	5.3	2.3	0.4340	41	5.7	ND	ND
18	5.3	2.3	0.4340	42	5.3	ND	ND
19	6.3	2.3	0.3651	43	5.3	ND	ND
20	5.4	3.0	0.5556	44	5.5	ND	ND
21	5.1	2.6	0.5098	45	5.7	2.5	0.4386

22	5.2	3.1	0.5962	46	5.5	2.8	0.5091
23	5.7	3.2	0.5614	47	6.0	2.7	0.4500
24	5.2	3.1	0.5962	48	5.4	2.9	0.5370

ND= NOT DETERMINED.

Since the procedure was done in duplicate, the average of each of the samples was calculated, and the results were compared by groups according to Table 2. Furthermore, the results were compared for each inhibitor used, to show the greatest glycation effect or, conversely, the greatest inhibitory effect on hemoglobin glycation.

In addition to evaluating the HbA1c values of each blood sample, fluorescence was also recorded at 320/460 nm and excitation / emission 355/460 nm. Since the procedure was done in duplicate, the average of each of the samples was calculated and each average obtained was plotted (Figure not shown). The results are shown in Tables 4 and Table 5; and the results of the averages of fluorescence are shown in Table 6.

Table 5: Fluorescence recorded in the range of 320-460 nm.

Sample Number	Fluorescence	Sample Number	Fluorescence	Sample Number	Fluorescence
1	104.50	17	89.67	33	89.11
2	93.05	18	92.09	34	89.24
3	97.49	19	93.08	35	92.86
4	91.96	20	87.73	36	91.74
5	90.76	21	88.42	37	137.60
6	92.95	22	86.05	38	ND
7	91.72	23	99.10	39	ND
8	93.37	24	103.00	40	ND
9	90.26	25	95.05	41	ND
10	86.59	26	95.44	42	ND
11	92.93	27	96.55	43	ND
12	94.10	28	90.92	44	ND
13	93.25	29	101.60	45	134.80
14	97.03	30	99.70	46	120.70
15	92.24	31	91.85	47	157.50
16	92.04	32	87.00	48	140.70

Table 6: Fluorescence recorded in the range of 355-460 nm.

Sample Number	Fluorescence	Sample Number	Fluorescence	Sample Number	Fluorescence
1	6.027	17	5.635	33	5.252
2	5.654	18	5.290	34	5.342
3	6.126	19	5.549	35	5.632
4	5.679	20	5.004	36	5.283
5	5.681	21	5.142	37	8.020
6	5.506	22	4.833	38	ND
7	5.667	23	6.086	39	ND
8	5.803	24	6.449	40	ND
9	5.060	25	5.998	41	ND
10	5.150	26	5.683	42	ND
11	5.699	27	5.793	43	ND
12	5.440	28	5.381	44	ND
13	5.473	29	6.392	45	7.337
14	5.967	30	6.076	46	6.830
15	5.262	31	5.415	47	9.190
16	5.504	32	5.295	48	7.889

4. DISCUSSION

The determination of glycated hemoglobin has been used as a marker of the degree of metabolic control in diabetic patients. Its percentage allows know how the blood glucose behavior has been up to three months before. So it has been considered a more reliable value than the same blood glucose determination. In this work, glycation caused by both glucose and ketone bodies; acetoacetate and acetone was studied. The effect of different inhibitors on hemoglobin glycation caused by these glycating substances was also evaluated. The concentrations of glycant agents used here were similar to those found in diabetic patients with poor metabolic control,[16] while the concentrations of the antiglycant substances were the same as those used in previous studies carried out in our Laboratory.[13] HbA1c levels were determined by an immunoassay method that allows evaluating the HbA1c concentration and its percentage compared to total hemoglobin.

During the process of adding acetone to the corresponding samples (Table 2), the instantaneous denaturation of plasma proteins was observed, in addition to the lysis of erythrocytes. One of the effects of acetone can be easily explained since acetone is less polar than water and interacts with membrane phospholipids causing their disaggregation. The observations made here could be extrapolated to *in vivo* situations where possible adverse effects of acetone could occur in patients with ketoacidosis, a condition in which patients have a characteristic odor when removing acetone during respiration.

The effects of acetone on hemoglobin glycation in intact erythrocyte could not be evaluated, however, direct effects on erythrocyte membrane disaggregation could be observed. These observations allow the hypothesis that low concentrations of acetone produced by spontaneous decarboxylation of acetoacetate could also affect the membrane integrity of cells other than blood cells and mitochondrial membranes.

Despite the fact that in a certain way the levels of acetone could be regulated by its reduction to isopropyl alcohol by alcohol dehydrogenase in the body.[17]

In Table 3, the samples treated with acetone were included and they are registered as not determined (ND), due to they were very viscous after acetone addition and incubation. Since acetone being less polar than water, it can form hydrogen bonds with other compounds with similar properties, therefore, it breaks the hydrogen bonds of plasma proteins and they lose their tertiary structure.

Table 4 shows the increase in glycated hemoglobin from baseline. In this table it can be seen that the samples containing glucose have an increase of about 1.5 times with respect to the initial concentration. In addition, no greater increase is observed in the control samples without inhibitor compared to the samples with inhibitor, except for L-arginine whose values are 0.9216 and 0.4423 increase, respectively. On the other hand, the highest value in this group corresponds to sample number 9, which contained glucose and aminoguanidine (Table 2).

Samples containing acetoacetate showed final HbA1c percentages lower than the initial values, so the results are inconsistent since final HbA1c values lower than the initial ones cannot be obtained. Furthermore, the results obtained from the controls without inhibitors show the same behavior as the rest of the group with acetoacetate.

Since acetoacetic acid has a pK of 3.58 and a concentration of 1.5 mg ml^{-1} was used, which have been reported in patients with ketoacidosis; the pH of the samples with acetoacetate decreases considerably, affecting the tertiary structure of proteins.

The effect of acetoacetate was not observed immediately during its addition to erythrocytes, but it was observed after 90 days of incubation, where the reported HbA1c values were not congruent and therefore the effects of acetoacetate can be observed in concentrations in those

that would be found in a patient with ketoacidosis. This effect could be enhanced by acetone formed by spontaneous decarboxylation of acetoacetate.

It could be seen that the percentage of glycated hemoglobin, which represents these results by glycation-inducing groups. Group 1 (samples 1-12; hemoglobin plus glucose) shows the highest percentage of HbA1c, with respect to the rest of the other groups. Group 2 (samples 13-24; hemoglobin plus acetoacetate), group 3 (samples 25-36; hemoglobin plus glucose plus acetoacetate) and group 4 (samples 37-48; hemoglobin plus acetone). In the case of group 2 a very low percentage was observed, in fact none exceeds 3% of HbA1c, due to the causes previously described.

On the other hand, group 3 shows a very similar behavior to group 1, with the exception of glycine. Since group 3 contained glucose and acetoacetate and the behavior resembles group 1. Glycation is attributed mainly to glucose, since the terminal amino group of hemoglobin reacted preferentially with the aldehyde group of glucose than the carbonyl groups of acetoacetate, because acetoacetate has acidic hydrogen atoms at carbon 2 of its structure and therefore the reaction between the amino group and acetoacetate would be of the acid-base type and not of the nucleophilic type, so the formation of a Schiff's base would not be favored in comparison with the reaction with glucose. Therefore, the effect of acetoacetate was irrelevant as a glycant substance under the conditions of this study.

In the case of group 4, the results are very poor and do not allow an analysis of the glycant effect of acetone on hemoglobin given the circumstances mentioned above.

On the effect of the different inhibitors used here, it could be seen that the behavior pattern with respect to the controls is similar since they have close values, except for L-arginine, where there is a small difference in aminoguanidine, where there was an increase in HbA1c compared to the control group. So it could be said that in this study aminoguanidine did not have a good inhibitory effect on glycation. A decrease in hemoglobin glycation with L-arginine is clearly observed compared to the control group. This confirms the antiglycant effect of L-arginine previously demonstrated in different studies in our Laboratory, due to the presence of the guanidinium group in its structure.

In Tables 5 and 6, the fluorescence results are shown. The fluorescence of samples 38 to 44 was not recorded due to the aforementioned circumstances.

The average of each sample made in duplicate was calculated, and the values obtained were plotted (Figure not shown), it was observed that the fluorescence values obtained had the same behavior with respect to the control samples; except in samples 37, 45 and 47. However, baseline fluorescence readings were made of blood samples from healthy volunteers where the fluorescence values at 320-460 nm and 355-460 (excitation-emission) were 75.117 and 3.766, respectively. Comparing these values with those obtained after incubation (Tables 5 and 6), an increase in fluorescence could be observed after incubation, which demonstrates the formation of fluorescent AGEs during incubation. However, the resulting fluorescence can not only be a product of HbA1c but also of other plasma proteins.

5. CONCLUSIONS

In conclusion, these results suggest that high concentrations of glucose produced glycation of hemoglobin in intact erythrocytes, which confirms the interaction between glucose and hemoglobin, serving as a reference parameter to compare the glycant effect of actoacetate and acetone. When blood samples were incubated in the presence of acetoacetate no relevant glycation of hemoglobin was observed, however, when acetone was added to blood samples, an inmediate denaturing effect on plasma proteins was observed. By interaction of acetone with phospholipids and proteins, disaggregation of erythrocyte membrane occurred. Inhibition of hemoglobin glycation caused by glucose was observed when L-arginine was added. However, no inhibitory effect on hemoglobin glycation by glycine, glycylglycine, aminoguanidine and urea was observed in the conditions of this study. The chemical structure of the fluorescent compounds remain to be investigated.

6. PERSPECTIVES

Based on these results, it is consider necessary perform additional *in vitro* studies, to evaluate the glycation process in short periods of time in order to know in detail the glycation pattern of hemoglobin under the previously described and standardized conditions, and also evaluate the membrane integrity of the erythrocyte.

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8. REFERENCES

1. Voet D, Voet JG. Biochemistry. 2nd Ed. John Wiley & Sons, Inc. New York, USA., 1995; 678-680.
2. Nelson DL, Cox MM. Lehninger Principles of Biochemistry. Worth Publishers. 3rd ed. New York, USA., 2000; 615-618.
3. Koorevaar G, Van Stekelenburg GJ. Mammalian acetoacetate decarboxylase activity: Its distribution in subfractions of human albumin and occurrence in various tissues of the rat. Clin. Chim. Acta., 1976; 71(2): 173-183.
4. https://web.archive.org/web/20070110024327/http://www-medlib.med.utah.edu/NetBiochem/FattyAcids/10_2.html
5. Musa-Veloso K, Likhodii SS, Cunnane SC. Breath acetone is a reliable indicator of ketosis in adults consuming ketogenic meals. Am. J. Clin. Nutr., 2002; 76(1): 65-70.
6. 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.
7. Nyenwe EA, Kitabchi AE. The evolution of diabetic ketoacidosis: An update of its etiology, pathogenesis and management. Metab.: Clin. Exp., 2016; 65: 507-521.
8. 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; 479-498.
9. Ramphul K, Joynauth J. An update on the incidence and burden of diabetic ketoacidosis in the U.S. Diabetes Care, 2020; 43(12): e196-e197.
10. Javed Khan M, Khan N, Ahmad N. Clinical profile of patients with diabetic ketoacidosis. WJPMR, 2020; 6(5): 91-94.
11. Lewis GD, Laufman AK, McAnalley BH, Garriott JC. Metabolism of acetone to isopropyl alcohol in rats and humans. J Forensic Sci., 1984; 29: 541–549.

12. Thornalley PJ, Langborg A, Minhas HS. Formation of glyoxal, methylglyoxal and 3-deoxyglucosone in the glycation of proteins by glucose. Biochem. J., 1999; 344(Pt 1): 109-116.
13. Ramachandra Bhat L, Vedantham S, Krishnan UM, Rayappan JBB. Methylglyoxal - An emerging biomarker for diabetes mellitus diagnosis and its detection methods. Biosens. Bioelectron, 2019; 133: 107-124.
14. Xie, J., García-Pérez, E. and Méndez, J.D.: *In vitro* glycation of hemoglobin by acetone and β-hydroxibutirate. World Appl. Sci. J., 2007; 2(2): 99-106.
15. Méndez JD, Leal LI. Inhibition of *in vitro* pyrraline formation by L-arginine and polyamines. Biomed. Pharmacother, 2004; 58: 598-604.
16. Méndez, J.D., Xie, J. and García-Pérez, E.: Urea inhibits the *in vitro* formation of fluorescent advanced glycation end products. World Appl. Sci. J., 2007; 2(2): 90-98.
17. Davidson J.K., Parker D.R. Monitoring of Boold and Urine Glucose and Ketone Levels; Clinical Diabetes Mellitus: A problem-Oriented Approach. Chap. 22, 3th. Ed. Italy, Thieme, 2000; 447,448.
18. Lewis GD, Laufman AK, McAnalley BH, Garriott JC. Metabolism of acetone to isopropyl alcohol in rats and humans. J. Forensic Sci., 1984; 29: 541–549.