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Glucosamine effects on platelet aggregation of type 2 diabetes mellitus patients*: in vitro* assays

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ABSTRACT

Hyperglycemia, insulin resistance, and endothelium dysfunction are related to platelet hyperactivity in type 2 diabetes mellitus (T2D) patients. Glucosamine (GlcN) has inhibitory effects on platelets of animals and healthy donors, but this role in platelets from T2D patients is unknown. The aim of this study was to evaluate the GlcN *in vitro* effects on platelet aggregation in T2D patients and healthy donors. Donors´ and T2D patients' samples were analyzed through flow cytometry, Western blot, and platelet aggregometry. Platelet aggregation was induced using ADP and thrombin, with or without GlcN, N-Acetyl-glucosamine, galactose, or fucose. GlcN inhibited ADP and thrombin-induced platelet aggregation, while the other carbohydrates did not. GlcN suppressed the second wave of ADP-induced platelet aggregation. No differences in the percent of inhibition of ADP-induced platelet aggregation by GlcN were found between donors and T2D patients, but this effect was significantly higher in healthy donors using thrombin as an agonist. In addition, GlcN increased protein O-GlcNAcylation (O-GlcNAc) in the platelets from T2D patients but not in healthy donors. In conclusion, GlcN inhibited the platelet aggregation induced by ADP and thrombin for both study groups and increased O-GlcNAc in platelets from T2D patients. Further studies are required to evaluate the possible use of GlcN as an antiplatelet agent.

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Introduction

Type 2 diabetes mellitus (T2D) is a chronic and degenerative disease that affects millions of people worldwide (1). T2D causes vascular alterations, of which atherosclerosis is the main complication representing the leading cause of morbidity and mortality among patients (2). Clinically, atherosclerosis manifests as ischemic heart disease, cerebrovascular disease, or peripheral arterial disease. In addition to vascular complications, patients with diabetes mellitus show hemostatic changes that increase the thrombosis risk. This prothrombotic state includes increased blood coagulation factors levels and decreases in fibrinolysis and natural anticoagulant activity (3). Among the procoagulant modifications, it is well known that platelets of T2D patients are dysfunctional, characterized by an increase in activation, aggregation, adhesion to endothelial cells, mean volume, and count, but a decrease in nitric oxide and prostacyclin response. Hyperglycemia, insulin resistance, inflammation, oxidative stress, and endothelial dysfunction in T2D patients are some factors that favor platelet dysfunction (4).

In the clinic, diabetic patients are treated with antiplatelet drugs due to the high risk of cardiovascular disease (5,6). This has allowed the development and search for new agents with better antiplatelet properties, including natural compounds.

Glucosamine (GlcN) is an amino sugar with a wide application in biomedical areas such as health care, food, and cosmetic industries (7). GlcN is a dietary supplement and one of the most consumed supplements worldwide (8). In the field of blood coagulation, GlcN exerts inhibitory activity on platelet aggregation of animal and healthy human donors (9-13). To date, it knows that primary amines inhibit platelets aggregation induced by various agonists (14,15); however, in the particular case of GlcN, this amino sugar could inhibit platelet aggregation in a different way that involves proteins O-GlcNAcylation (O-GlcNAc), which in the last years has been associated with diabetes complications such as nephropathy and cardiovascular disease (16). Independently on the mechanism and their known antiplatelet effects, there is no evidence about the effects of GlcN and their magnitude on platelets from T2D patients. Therefore, this study aimed to evaluate by *in vitro* assays if GlcN exerts inhibitory effects on platelet aggregation of T2D patients.

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Materials and Methods

Patient selection

A Physician handled the selection of donors and T2D patients. Blood samples were drawn at Universidad Autónoma "Benito Juárez" de Oaxaca (UABJO). Platelet analyses and quantification of clinical and biochemical markers were done at Facultad de Medicina y Cirugía of the same institution. The donors´ group consisted of individuals with no known diseases, apparently healthy. We included adult donors and T2D patients, men and women, without a diagnosis of infectious or autoimmune disease, who had not ingested drugs that altered the platelet activity in the last 15 days before drawing samples and after donors and T2D patients gave their informed consent. All patients had at least one year with a diagnosis of T2D and were included if their glucose levels were ≥ 126 mg/dl on the day of sampling. Pregnancy, clinical history of hemorrhage or thrombosis, and a platelet count of less than 150,000/µl were criteria to exclude donors and diabetic patients from the study. This research was approved by the Research Ethics Committee of UABJO (Number: 22PE1001FO).

Sample collection

From each donor and T2D patient, 10 ml of blood was used and distributed in 3 vacuum tubes with 3.2% trisodium citrate (vol: vol = 1: 9) (Na Citrate, BD Vacutainer, Franklin Lakes, NJ, USA) and one tube without anticoagulant (SST, BD Vacutainer, Franklin Lakes, NJ, USA). Blood samples were drawn after a 12-hour fast and left to rest for 10 minutes before being centrifuged at 800 g for 10 minutes to get the platelet-rich plasma (PRP). The PRP was employed to evaluate platelet aggregation with ADP. Then, washed platelets were obtained according to a previous study (12). This procedure was used to assess platelet aggregation with thrombin. Sera was obtained after centrifuging 5 ml of blood at 2000 g/10 min to perform blood analysis.

Sample size

It was determined using the results of donors´ percentage of platelet aggregation (n = 60). This calculation was based on comparing means and considering an expected standard deviation of 18 %, a mean of 80 %, and a decrease of 20 % of thrombin-induced platelet aggregation by GlcN. Based on the above and considering a value of $\alpha = 0.05$ and $1\text{-}\beta = 0.8$, the estimated sample size for statistical analysis was 10 individuals per group.

Blood analysis

All samples were analyzed using diagnostic kits in the Synchron LX 20 chemical analyzer (Beckman Coulter, Fullerton, CA, USA) to obtain serum levels of glucose, triglycerides, cholesterol, cholesterol in high-density lipoprotein (HDL-cholesterol), low-density lipoprotein cholesterol (LDL-cholesterol), very low-density lipoprotein cholesterol (VLDL-cholesterol), serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), dehydrogenase lactic acid (LDH), alkaline phosphatase (AP) and gamma-glutamyl transferase (GGT), in addition to the levels of urea, urea nitrogen, and creatinine. The atherogenic index was calculated as follows: total cholesterol / HDL cholesterol.

P-selectin expression

P-selectin expression was measured according to the manufacturer and published recommendations with some changes to quantify and compare the number of activated platelets between groups. In brief, washed platelets were incubated with PBS or thrombin 0.5 U/ml at 37°C for 1 min and fixed with paraformaldehyde 1% for 15 min at room temperature. Then 10 µl of a mouse anti-human P-selectin antibody [BioLegend, FITC anti-human CD62P (P-Selectin) antibody, San Diego, CA, USA] was added to a solution of 200,000 platelets/µl for incubation of 2 h at 37°C on darkness conditions. Ten thousand events were acquired to determine the percentage of activated platelets. Samples were analyzed in Attune NxT Flow cytometer using Flowjo software v.10.

Platelet aggregation assay

This procedure was evaluated *in vitro* using a commercial aggregometer (Chronolog, Havertown, PA, USA). ADP (Sigma-Aldrich, Adenosine 5 diphosphate, St. Louis, MO, USA) and thrombin (Diagostica Stago, fibrinogen reagent, Asnières, France) concentrations to evaluate platelet aggregation were defined using increasing concentrations of both agonists. The platelet count was adjusted to 200,000 platelets/µl. PBS buffer (pH = 7.4) was used as a test blank. Aggregation assays were measured before (basal) and after the incubation of platelets at different GlcN (Sigma-Aldrich, glucosamine hydrochloride, St. Louis, MO, USA) concentrations (1, 5, 10 and 20 mM) and incubation times (0.5, 1.0, 2.0 and 5.0 min). Platelet auto-aggregation before adding ADP or thrombin was an exclusion criterion for samples. The percentage of platelet aggregation was determined until it reached the maximum level of light transmittance, often obtained 7 min after starting the assay. Carbohydrates N-acetylglucosamine (GlcNAc; Sigma-Aldrich,), galactose (Gal; Sigma-Aldrich, D (+) galactose, St. Louis, MO, USA), and fucose (Fuc; Sigma-Aldrich, L (-) Fucose, St. Louis, MO), USA) were incubated with platelets at a final concentration of 20 mM to evaluate the specificity of GlcN effects on platelet aggregation.

Western blot analysis

Protein extraction and quantification were performed according to a previous study (12). Thirty micrograms of protein were used for SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Bio-Rad, nitrocellulose membrane, Hercules, CA, USA) using a semi-wet chamber (Trans-blot Transfer Medium, Bio-Rad, CA, USA). To avoid non-specific antibody binding, 5% albumin was used in Tris-buffered saline. The membrane was incubated overnight at 4°C with the anti-O-linked N-acetylglucosamine antibody, RL2 (Abcam Cambridge, United Kingdom). Then membranes were washed using 0.5% tween in saline, buffered 3 times for 5 min, and incubated with sheep anti-mouse IgG conjugated with horseradish peroxidase (Abcam, Sheep Anti-Mouse IgG H and L (HRP)) at room temperature for 120 min. The O-GlcNAcylated proteins were visualized in the membrane after adding hydrogen peroxide to a solution composed of diaminobenzidine and nickel chloride in buffer Tris pH 7.4. The membranes were scanned and subjected to quantitative densitometric analysis using Image J software 1.53e (17). The analysis represents the folds of

each group vs. the donor group without GlcN.

Statistical analysis

Results were analyzed using SigmaPlot Version 12.0 (Systat Software, San Jose, CA). Descriptive statistics was used to analyze the general characteristics of donors and T2D patients. The blood tests and western blot analysis results were expressed as means ± standard deviation. Platelet aggregation was expressed as a percentage as the mean ± standard error of the mean (SEM). For all statistical analyses, we performed normality and equal variance tests for all statistical analysis to choose between parametric and non-parametric tests. The differences between the values of platelet aggregation at the different experimental conditions of concentration and incubation time with the other carbohydrates were determined by the Kruskal Wallis test or One Way-ANOVA. To determine the differences between the percentages of inhibition of GlcN in ADP vs. thrombin, the Mann-Whitney U test or Student's t-test was used. The same tests were used to compare the results obtained from the blood analysis, the platelet aggregation tests, and the western blot analysis between donors and T2D patients. A p-value ≤ 0.05 was considered significant.

Results

Thirty samples for each study group were analyzed. The mean age, weight, and height of donors were 34.1 years (range: 18 - 57 years), 67.9 Kg (range: 42 - 89 Kg), and 1.6 m (range: 1.5 - 1.8 m), respectively. By contrast, the age, weight, and height of T2D patients were 47.4 years (range: 35 -64), 66.7 kg (range: 45 - 88 kg), and 1.5 m (range: 1.4 - 1.7 m), respectively. None of the donors and diabetic patients had obesity; the mean body mass index was 25.1 kg/m^2 (range: 18.0 – 28.2 kg/m^2) in the donors and 26.5 kg/m^2 (range: 20.3 - 27.8 kg/m^2) in diabetic patients. Forty percent of T2D patients were hypertensive.

Biochemical markers in donors and T2D patients

Clinical data and blood tests showed that donors and T2D patients had no renal or hepatic alterations; however, both groups had some alterations in triglycerides ($p < 0.05$) and VLDL-C ($p < 0.05$) determinations, as shown in Table 1. As expected, the principal alteration was glucose concentration, which was higher in the T2D patients (167.8 ± 19.52 mg/dl) compared to donors (84 ± 9.05 mg/dl); $p < 0.05$. Also, triglycerides (189.7 ± 92.5 mg/dl vs. 149.7 ± 71.6 mg/dl) and VLDL-C (38.0 ± 18.4 mg/dl vs. 30.1 ± 14.4 mg/dl) levels were higher in T2D patients than in donors. For the rest of the biomarkers, there were no significant differences between the two study groups (Table 1).

P-selectin expression in donors and T2D patients

At basal conditions, the expression of P-selectin was 11.0 ± 3.8 % vs. 16.0 ± 6.6 % in platelet from donors and T2D patients, respectively. After thrombin (0.5 IU/ml) activation, the expression increased to 67 ± 6.8 % for platelets from donors and 72 ± 7.7 % for T2D patients. For the basal ($p = 0.103$) and post-thrombin results ($p = 0.289$), no significant changes were observed in the expression of P-selectin between groups.

Thrombin and ADP induced platelet aggregation in donors and T2D patients

Platelet aggregation was evaluated using thrombin 0.2, 0.5, and 1.0 IU/ml and ADP 1.2, 2.4, and 5.0 μM. Platelets were incubated at 37°C for 1 min. Thrombin concentrations of 0.5 and 1.0 IU/ml induced the highest platelet aggregation in both groups, while 5 μM of ADP induced the maximum response. For thrombin, a concentration of 0.5 IU/ml was used to induce platelet aggregation for the subsequent assays using GlcN; for ADP, a concentration of 2.4 μM was used due to the presence of the two characteristic waves of platelet aggregation. This effect did not occur using ADP 5 μM. No differences were observed for

Table 1. Results of blood analysis in donors and T2D patients.

	Donors (Mean ± SD)	T2D (Mean ± SD)	RV
Glucose	84.0 ± 9.05	167.8 ± 19.52*	70 – 100 mg/dL
Urea	25.5 ± 9.6	31.1 ± 18.1	15-47 mg/dL
Ureic nitrogen	11.9 ± 4.4	14.6 ± 8.4	7-25 mg/dL
Creatinine	0.7 ± 0.2	0.7 ± 0.6	0.60-1.20 mg/dL
Triglycerides	149.7 ± 71.6	189.7 ± 92.5*	35-150 mg/dL
Cholesterol	158.8 ± 42.7	155.3 ± 12.4	150-200 mg/dL
HDL-C	37.7 ± 8.3	39.3 ± 9.0	≥ 40 mg/dL
LDL-C	90.7 ± 34.1	89.8 ± 27.87	< 100 mg/dL
VLDL-C	30.1 ± 14.4	38.0 ± 18.4*	2-30 mg/dL
Atherogenic index	4.2 ± 0.8	4.1 ± 0.9	≥ 4.0
SGOT	24.2 ± 9.47	22.1 ± 7.52	14.0 – 78.0 U/dL
SGPT	21.5 ± 12.01	20.0 ± 12.91	7.0 – 52.0 U/dL
AP	69.1 ± 20.58	81.3 ± 21.13	34 – 104 U/dL
LDH	158.4 ± 56.15	167.0 ± 77.28	140 – 271 U/dL
GGT	23.1 ± 14.43	33.1 ± 22.58	9.0 – 64 U/dL
HbA1c	---	7.1 ± 0.74	≤ 5.6 %

AP: alkaline phosphatase; GGT: gamma-glutamyl transferase; HbA1c: hemoglobin A1c; HDL-C: High-density lipoprotein cholesterol; LDH: lactate dehydrogenase; LDL-C: low-density lipoprotein cholesterol; SGOT: serum glutamic oxalacetyc transferase; SGPT: serum glutamic pyruvate transferase; VLDL-C: very low-density lipoprotein-cholesterol *: $p<0.05$ between groups.

Table 2. Effect of agonists concentration on platelet aggregation in donors and T2D patients.

	Platelet aggregation (%)		
Agonist	**Donors**	**T2D**	**p**
Thrombin (0.2 IU/ml)	53.5 ± 3.2	60.1 ± 3.8	0.061
Thrombin (0.5 IU/ml)	79.4 ± 3.5	86.5 ± 2.8	0.278
Thrombin (1.0 IU/ml)	83.2 ± 2.9	87.4 ± 2.4	0.238
ADP (1.2 µM)	45.0 ± 2.6	55.1 ± 2.9	0.057
ADP (2.4 µM)	81.3 ± 3.6	80.4 ± 3.0	0.210
ADP (5.0 µM)	90.3 ± 2.3	88.2 ± 2.1	0.312

Results are expressed as the mean ± SEM. *: p<0.05 between groups.

platelet aggregation results with thrombin and ADP between donors and T2D patients (Table 2).

GlcN inhibited thrombin- and ADP-induced platelet aggregation

Tables 3 and 4 show the results of all platelet aggregation assays using GlcN. Four concentrations of GlcN were evaluated (1.0, 5.0, 10.0 and 20.0 mM) in platelets from donors (n=10) and T2D patients (n=10). Platelets were activated with thrombin (0.5 U / ml) or ADP (2.4 µM) at different incubation times (0.5, 1.0, 2.0 and 5.0 min). Baseline platelet aggregation with thrombin tends to be higher in patients with diabetes than in donors (Table 3), however, the difference was not significant. Table 3 shows that GlcN decreases platelet aggregation with thrombin in donors and TD2 patients at the concentration of 20 mM, except at 5 min of incubation for diabetic patients. The decrease in platelet aggregation was greater in donors than in TD2 patients regardless of incubation time.

For ADP, the effect of GlcN on platelet aggregation was observed from the 5 mM sugar concentration and incubation times of 2.0 and 5.0 minutes in both study groups (Table 4). At the 10 mM GlcN concentration, platelet aggregation with ADP in both study groups decreased significantly compared to the baseline determination, regardless of incubation time. However, between patients and

Table 3. Concentration-dependent effects of GlcN on thrombin-induced platelet aggregation in donors and T2D patients.

	Percentage of platelet aggregation in donors				
	GlcN concentration				
Incubation time (min)	**0 mM**	**1.0 mM**	**5.0 mM**	**10.0 mM**	**20.0 mM**
0.5	81 ± 5.6	74 ± 3.6	78 ± 5.8	77 ± 2.9	#43 ± 3.6
1.0	77 ± 4.2	83 ± 3.9	86 ± 4.3	80 ± 3.4	#54 ± 4.1
2.0	80 ± 4.5	82 ± 4.1	83 ± 5.1	81 ± 4.3	#38 ± 5.6
5.0	83 ± 4.9	76 ± 4.6	82 ± 4.0	82 ± 5.2	#68 ± 3.8
	Percentage of platelet aggregation in T2D patients				
0.5	89 ± 4.8	80 ± 5.4	77 ± 5.7	78 ± 3.3	#68 ± 3.9*
1.0	87 ± 5.0	81 ± 4.6	89 ± 4.7	78 ± 3.7	#64 ± 4.2*
2.0	85 ± 4.3	81 ± 4.5	78 ± 6.0	79 ± 3.2	#56 ± 4.5*
5.0	88 ± 4.2	80± 5.0	83 ± 4.2	83 ± 5.4	85 ± 5.2*

Results are expressed as the mean ± standard error of the mean. *: p<0.05 between donors and T2D patients; #: p<0.05: basal percentage of platelet aggregation (GlcN 0 mM) vs. percentage of platelet aggregation post-GlcN 1, 5, 10 and 20 mM.

Table 4. Concentration-dependent effects of GlcN on ADP-induced platelet aggregation in donors and T2D patients.

	ADP-induced platelet aggregation in donors				
	GlcN concentration				
Incubation time (min)	**0 Mm**	**1 mM**	**5 mM**	**10 mM**	**20 mM**
0.5	78 ± 3.3	80 ± 4.1	70 ± 5.6	#27 ± 3.2	#1 ± 2.0
1.0	77 ± 4.2	76 ± 5.2	75 ± 4.5	#29 ± 3.8	#3 ± 2.1
2.0	80 ± 2.7	76 ± 2.9	#58 ± 6.0	#14 ± 6.2	#1 ± 2.2
5.0	79 ± 3.8	70 ± 3.6	#41 ± 5.7	#35 ± 3.3	#1 ± 1.8
	ADP-induced platelet aggregation in T2D patients				
0.5	78 ± 5.1	82 ± 2.8	75 ± 4.2	#30 ± 3.5	#1 ± 1.7
1.0	81 ± 4.2	74 ± 3.1	72 ± 4.9	#36 ± 4.4	#3 ± 2.4
2.0	80 ± 3.9	74 ± 4.5	#64 ± 5.8	#25 ± 3.9*	#1 ± 2.0
5.0	82 ± 4.6	76 ± 3.4	#62 ± 6.1*	#44 ± 5.4*	#3 ± 5.4

Results are expressed as the mean ± standard error of the mean. *: p<0.05 between donors and T2D patients; #: p<0.05: basal percentage of platelet aggregation (GlcN 0 mM) vs. percentage of platelet aggregation post-GlcN 1, 5, 10 and 20 mM.

donors, the inhibition of platelet aggregation by 10 mM GlcN was higher in donors than in T2D patients at incubation times of 2 and 5 minutes (Table 4). Finally, with 20 mM GlcN the highest inhibition of platelet aggregation with ADP was observed both in donors and T2D patients, without observing significant differences between the results of both groups (Table 4). Since 20 mM GlcN showed the highest inhibitory effect, this concentration was used for the following platelet aggregation assays.

To calculate the percentage of inhibition of ADP and thrombin-induced platelet aggregation by the effect of GlcN, platelets were incubated with GlcN 20 mM for 0.5 min.

Percentage of inhibition of platelet aggregation by GlcN in donors and T2D patients

The percentage of inhibition by GlcN on platelet aggregation was calculated with 30 samples from donors and T2D patients. This value was calculated with the following equation: % Inhibition = 100 - [(% platelet aggregation post-GlcN / % platelet aggregation basal) x 100]. For donors, the GlcN decreased the platelet aggregation from 80.7 ± 3.8 % in baseline conditions to 39.3 ± 4.1 % ($p < 0.5$) using thrombin as an activator and from 87.3 ± 4.6 % to 54.9 ± 3.8 % in T2D patients ($p < 0.05$) (Table 5). When the ADP was employed, GlcN decreased the platelet aggregation from 79.3 ± 4.2 % to 1.5 ± 2.5 % in donors ($p < 0.05$), while in T2D patients, platelet aggregation decreased from 82.4 ± 3.4% to 2.2 ± 2.9 % ($p = 0.008$) (Table 5). Thus, the percentage of inhibition of platelet aggregation with thrombin was 51.3 ± 4.7% in donors and 37.1 ± 4.3% in T2D patients, and 98.1 ± 3.9% in donors and 97.3 ± 4.2% in T2D patients when platelets were activated with ADP. This inhibition was higher for donors than for T2D patients when platelets were activated with thrombin ($p = 0.034$), but not with ADP ($p = 0.821$) (Table 5). Figure 1 shows schematic platelet aggregation curves from donors and T2D patients using both agonists at basal or post-GlcN, and a graphic showing the comparisons of the percentage of inhibition of platelet aggregation between groups.

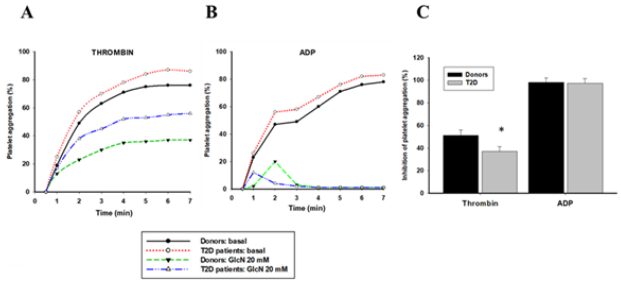

Figure 1. Platelet aggregation and percentage of inhibition by GlcN. A and B) Schematic platelet aggregation curves from donors and T2D patients using thrombin or ADP at basal (lines black and red) or post-GlcN (2.0 min /37°C, lines blue and green). C) Percentage of inhibition of platelet aggregation with thrombin and ADP for both study groups. Bars represent the mean ± standard error of platelet aggregation measured at the end of the assay. $p<0.05$ between groups.

To evaluate the specificity of GlcN to inhibit platelet aggregation, platelets were incubated with GlcNAc, Gal, and Fuc at the same concentration (20 mM), and incubation time was employed for GlcN. These carbohydrates did not decrease platelet aggregation induced with ADP and thrombin, so the percentage of inhibition of platelet aggregation was not calculated (Table 5).

O-GlcNAc shows slight differences between the platelets of donors and T2D patients

Western Blot analysis revealed a pattern of 12 to 15 bands of O-GlcNAc proteins at a range of 20 to 150 kDa in platelets from donors and T2D patients (Figure 2A). In platelets from T2D patients, two bands of major intensity (72 and 68 kDa) were observed at basal condition (-GlcN) compared to donors. For the 68-kDa band, GlcN did not modify protein O-GlcNAc in the platelets from both study groups; however, for the 72-kDa band, the amino carbohydrate increased the glycosylation in T2D patients` platelets, but not in donors (Figure 2B).

Table 5. Effects of GlcN and other sugars on platelet aggregation and inhibition percentage in donors and T2D patients.

	Donors (n=30)		T2D patients (n=30)		P
	Platelet aggregation (%)	% Inhibition	Platelet aggregation (%)	% Inhibition	
Thrombin-induced platelet aggregation					
Basal	80.7 ± 3.8		87.3 ± 4.6		0.437
GlcN (20 mM)	39.3 ± 4.1*	51.3 ± 4.7#	54.9 ± 3.8*	37.1 ± 4.3	0.047
GlcNAc (20 mM)	75.1 ± 3.9	-	83.7 ± 3.6	-	0.211
Gal (20 mM)	74.8 ± 6.5	-	80.1 ± 4.5	-	0.128
Fuc (20 mM)	78.1 ± 5.4	-	81.6 ± 5.4	-	0.064
ADP-induced platelet aggregation					
Basal	79.3 ± 4.2		82.4 ± 3.4		0.287
GlcN (20 mM)	1.5 ± 2.5*	98.1±3.9	2.2 ± 2.9*	97.3±4.2	0.315
GlcNAc (20 mM)	83.4 ± 4.6	-	84.7 ± 4.1	-	0.521
Gal (20 mM)	81.8 ± 4.3	-	85.6 ± 5.2	-	0.312
Fuc (20 mM)	80.7 ± 3.8	-	84.4 ± 4.3	-	0.226

Results are expressed as the median ± standard error. P values in the right raw correspond to the comparison of the percentage of platelet aggregation between donors and T2D patients; *: $p<0.05$ basal percentage of platelet aggregation vs. percentage of platelet aggregation post-GlcN, GlcNAc, Gal and Fuc. #: $p<0.05$, percentage of inhibition of platelet aggregation between donors and T2D patients.

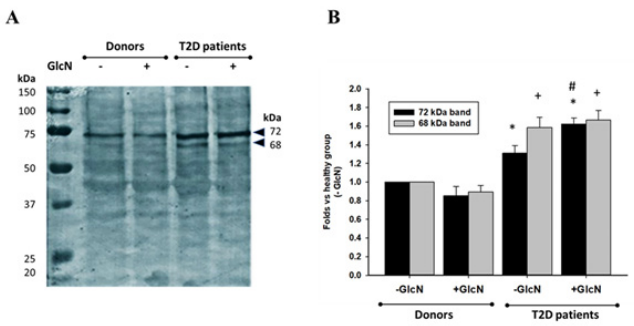

Figure 2. Effect of GlcN on O-GlcNAc Western blot in donors and T2D patients. A) After GlcN platelets incubation, two principal bands (72 and 68 kDa) were seen on the O-GlcNAc western blot in both study groups. B) Western blot analysis of these bands. Data are expressed as mean ± standard error. The significant difference of * group T2D-GlcN, + group T2D+GlcN vs. donors-GlcN, respectively, and # group T2D+GlcN vs T2D-GlcN ($P \leq 0.05$).

Discussion

The effects of GlcN on platelet aggregation have been known for 40 years. Bertram *et al.* (1981) demonstrated *in vitro* that GlcN inhibits platelet aggregation with ADP, epinephrine, and collagen using human platelets (9); these results were corroborated later by other authors (10-13). However, its effects on the platelets of T2D patients had never been evaluated.

In the first step, the differences in platelet activation were evaluated through the knowledge of p-selectin expression followed by the assays of platelet aggregation between donors and T2D patients using different concentrations of platelet agonists. A higher number of platelets from diabetic patients expressed P-selectin than donors, but no statistical difference was found. Also, we found that donors and diabetic patients showed similar results with a tendency to have higher platelet aggregation results in samples from diabetic patients compared to donors; however, for both agonists, the differences were not significant. Therefore, these results could not demonstrate a hyperaggregability state in T2D patients.

On the other hand, this *in vitro* study demonstrated for the first time that GlcN inhibits in a dose-dependent manner the platelet aggregation induced with thrombin and ADP in samples of T2D patients, with more pronounced effects on ADP response, just as it was described in healthy donors (10,12). This effect was specific to GlcN because platelet aggregation did not change in the presence of other monosaccharides (GlcNAc, Gal, and Fuc, Table 5).

Although GlcN inhibited platelet aggregation with ADP and thrombin in both study groups, we highlight that the inhibition of thrombin-induced platelet aggregation with GlcN 20 mM was significantly higher in platelets from donors than in T2D patients. In contrast, the percentage of inhibition of ADP-induced platelet aggregation was similar for both study groups. Also, we highlight that GlcN affects granular secretion since the second wave of platelet aggregation was abolished. These findings suggest that the inhibition of ADP signaling is the mechanism by which GlcN inhibits platelet aggregation affecting ADP-induced granular secretion and the release of ADP induced by the action of thrombin. Similar results to ours were found previously by Hua *et al.*, in 2004, who established a possible inhibitory mechanism of GlcN on platelet aggregation induced by ADP and thrombin (10). Briefly, they demonstrated that GlcN inhibits the initial and second phases of platelet aggregation, with a prominent effect on the latter, reducing the maximum number of high (45% inhibition) and low (80% inhibition) affinity binding sites for ADP. That is, GlcN more strongly inhibited the P2Y12-mediated secondary phase of platelet aggregation than the P2Y1-mediated initial phase of platelet aggregation in response to ADP. In the same study, platelets were stimulated with low concentrations of thrombin (0.1 ~ 1 U/ml) to evaluated the secretion-dependent platelet aggregation induced by the agonist. They found that GlcN at 10 mM but not 1 mM inhibited the thrombin-induced platelet aggregation by 30-35%. Therefore, the authors suggested that GlcN could antagonize the action of not only the exogenous ADP but also the endogenous ADP released from platelets by thrombin stimulation. In addition, they speculated that GlcN partially inhibited the thrombin-induced platelet aggregation, because the level of extracellularly released ADP was low when platelets were stimulated with low concentrations of thrombin. The stimulation was possibly mediated via the receptors P2Y1 rather than P2Y12 (10). Besides the effects of GlcN on platelet aggregation, the study revelated that the inhibiton of ADP-binding to the receptors by GlcN, moderately suppresses the production of thromboxane A2, calcium movilization and phosphorylation of Syk (10).

Although the mechanism above could explain our results, in this study, we evaluated another possible one that involves the changes in platelet O-GlcNAc. This glycosylation consists of the binding of GlcNAc to Ser or Thr residues on cytoplasmic, nuclear, or mitochondrial proteins that regulate fundamental processes for cells (18). In 2008, using two animal models of DM, it was demonstrated that the increase in platelet O-GlcNAc does not relate to platelet hypersensitivity to thrombin (19). Besides, in 2017, our group showed slight changes in platelet O-GlcNAc (12) that accompanied the inhibition of platelet aggregation by GlcN. However, we point out that GlcN effects on O-GlcNAc were relatively discrete and often difficult to appreciate.

The present study demonstrated changes in O-GlcNAc between platelets from donors and T2D patients, with a higher expression in some platelet proteins from diabetic patients. Nevertheless, the incubation with GlcN induced changes in this glycosylation only in platelets from T2D patients (Figure 2). Concerning these findings, we hypothesize that hyperglycemia in diabetic patients increases the flow of glucose into HBP in both the platelet's precursors, the megakaryocytes, and directly on platelets in the bloodstream. Consequently, platelets from T2D patients showed increased protein O-GlcNAc and a better response to GlcN because the O-GlcNAc transferase enzyme is active and likely overexpressed. On the other hand, since GlcN did not increase O-GlcNAc in donor platelets but did in platelets from T2D patients, and because GlcN inhibits platelet aggregation with thrombin in a better way in donors than in T2D patients, these results suggest that this glycosylation is not related to the inhibitory effect of GlcN on platelet aggregation. Therefore we assume that GlcN inhibits the ADP- and thrombin-induced platelet aggregation in donors and T2D patients by the mechanism proposed by Hua *et al.,* (10).

Regardless of the mechanism of action, GlcN could be helpful in patients with an unfavorable response to ADP antagonists used in the clinic, as it happens with patients treated with clopidogrel (20). Nevertheless, the evidence presented in this study was obtained from *in vitro* assays using high concentrations of GlcN that could hardly be achieved in diabetic patients. However, in healthy donors, oral administration of GlcN at a dose of 1.5 g/day decreased ADP-induced platelet aggregation by up to 30%, and serum levels of GlcN reached 0.020 ± 0.006 mM (10). Therefore, there is a possibility that GlcN has similar effects in diabetic patients, with no apparent adverse outcomes (21,22).

In conclusion, for the first time, we evidenced the *in vitro* inhibitory action of GlcN on platelet aggregation of diabetic patients, mainly affecting the response to ADP. This effect was similar between donors and T2D patients, so further studies should evaluate the possible therapeutic effect of GlcN on platelet aggregation in diabetic patients. However, we still have to explain in other studies the difference observed in the inhibitory effect of GlcN on the thrombin-induced platelet aggregation between study groups. Finally, we highlight that although the mechanism involved in the inhibition of platelet aggregation by GlcN may not be related to O-GlcNAc, the use of O-GlcNAc transferase enzyme inhibitors will allow us to know about the role of O-GlcNAc on the biology of platelets.

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Interest of conflict
The authors declare not to have any interest conflicts.

Author's contribution
Gallegos-Velasco IB and Hernandez Cruz PA designed the experiments and reviewed the article writing. Fernandez-Rojas B analyzed the results and reviewed the article writing. Perez-Acevedo MA carried out the experiments and analyzed the results. Hernandez-Juarez J designed and carried out the experiments, analyzed the results, wrote and reviewed the article writing.

References

1. International Diabetes Federation. IDF Diabetes Atlas. 8th ed. (International Diabetes Federation; Brussels, Belgium), 2017. Online version.
2. Petrie JR, Guzik TJ, Touyz RM. Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms. Can J Cardiol 2018;34:575–584.
3. Sobczak AIS, Stewart AJ. Coagulatory Defects in Type-1 and Type-2 Diabetes. Int J Mol Sci 2019;20:6345.
4. Kaur R, Kaur M, Singh J. Endothelial dysfunction and platelet hyperactivity in type 2 diabetes mellitus: molecular insights and therapeutic strategies. Cardiovasc Diabetol 2018;17:121.
5. Majithia A, Bhatt DL. Antiplatelet Therapy in Diabetes. Endocrinol Metab Clin North Am 2018;47:223-235.
6. Paven E, Dillinger JG, Bal Dit Sollier C, Vidal-Trecan T, Berge N, Dautry R, et al. Determinants of aspirin resistance in patients with type 2 diabetes. Diabetes Metab 2020;46:370-376.
7. Ma Q, Gao X. Categories and biomanufacturing methods of glucosamine. Appl Microbiol Biotechnol 2019;103:7883-7889.
8. Moon JM, Finnegan P, Stecker RA, Lee H, Ratliff KM, Jäger R, et al. Impact of Glucosamine Supplementation on Gut Health. Nutrients 2021;13:2180.
9. Bertram J, Ragatz BH, Baldwin W, Iatrides PG. The effects of glucosamine on platelet aggregation. Thromb Res 1981;23:301-307.
10. Hua J, Suguro S, Iwabuchi K, Tsutsumi-Ishii Y, Sakamoto K, Nagaoka I. Glucosamine, a naturally occurring amino monosaccharide, suppresses the ADP-mediated platelet activation in humans. Inflamm Res 2004;53:680-688.
11. Lu-Suguro JF, Hua J, Sakamoto K, Nagaoka I. Inhibitory action of glucosamine on platelet activation in guinea pigs. Inflamm Res 2005;54:493-499.
12. 12. Hernández-Juárez J, Gallegos-Velasco IB, Pérez-Acevedo MA, Pérez-Cervera Y, Solorzano-Mata C, Pina-Canseco MS et al. Glucosamine inhibits platelet aggregation and modifies protein O-GlcNAcylation. J Nat Biol 2017;8:86-98.
13. Lin PC, Jones SO, McGlasson DL. Effects of glucosamine and Celadrin on platelet function. Clin Lab Sci 2010;23:32-36.
14. Ganguly P, Bradford HR. Inhibition of platelet aggregation by primary amines. Evidence for a possible role of membrane-associated calcium. Biochim Biophys Acta 1982;714:192-199.
15. Kinlough-Rathbone RL, Packham MA, Mustard JF. Effect of amino sugars on platelet aggregation and on fibrinogen binding. Thromb Haemost 1984;52:75–80.
16. Peterson SB, Hart GW. New insights: A role for O-GlcNAcylation in diabetic complications. Crit Rev Biochem Mol Biol 2016;51:150-161.
17. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature Methods 2012;9:671-675.
18. Nie H, Yi W. O-GlcNAcylation, a sweet link to the pathology of diseases. J Zhejiang Univ Sci B. 2019;20:437-448.
19. Crawford GL, Hart GW, Whiteheart SW. Murine platelets are not regulated by O-linked beta-N-acetylglucosamine. Arch Biochem Biophys 2008;474:220-224.
20. Guirgis M, Thompson P, Jansen S. Review of aspirin and clopidogrel resistance in peripheral arterial disease. J Vasc Surg 2017;66:1576–1586.
21. Ma H, Li X, Zhou T, Sun D, Liang Z, Li Y, et al. Glucosamine Use, Inflammation, and Genetic Susceptibility, and Incidence of Type 2 Diabetes: A Prospective Study in UK Biobank. Diabetes Care 2020;43:719–725.
22. Gommans YMM, Runhaar J, Jacobs ML, Bierma-Zeinstra SMA. The Effect of Prolonged Glucosamine Usage on HbA1c Levels and New-Onset Diabetes Mellitus in Overweight and Obese Middle-Aged Women. Am J Med 2017;130:731-737.