Methods: A new protocol for *in vitro* red blood cell glycation

Marcus V. Batista da Silva[1], Analía Alet[1], Horacio Castellini[2], Bibiana Riquelme[1,3]

[1]Área Física, Facultad Cs. Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario. Suipacha 535, 2000 Rosario, ARGENTINA.

e-mail: mbatista@fbioyf.unr.edu.ar, aalet@fbioyf.unr.edu.ar, briquel@fbioyf.unr.edu.ar

[2] Departamento de Física, Facultad Cs. Exactas, Ingeniería y Agrimensura, Universidad Nacional de Rosario. Av. Pellegrini 250, 2000 Rosario, ARGENTINA.

e-mail: hcaste@fceia.unr.edu.ar

[3] Grupo de Física Biomédica, IFIR (CONICET-UNR). Bv. 27 de febrero 210 bis, 2000 Rosario, ARGENTINA.

e-mail: riquelme@ifir-conicet.gov.ar

Corresponding author

Dra. Analía Alet

Facultad Cs. Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario.

Departamento Química-Física.

Suipacha 535, 2000 Rosario, Argentina.

e-mail: analia_alet@yahoo.com.ar

ABSTRACT

During diabetes, the characteristic hyperglycemia can induce red blood cell glycation. Several researchers have proposed different protocols to perform an *in vitro* model to study this phenomenon. In this article, some of the most important *in vitro* glycation protocols available in the bibliography were compared to each other. The incubation parameters as the suspension medium, glucose concentration, red blood cell concentration, time, and temperature were analyzed. Also, several assays were carried out in our laboratory, and glycated hemoglobin, erythrocyte aggregation and viscoelasticity were determined for the protocol validation. Based on the bibliographic analysis and our experimental results, an optimal protocol for in vitro glycation of red blood cells is presented.

KEYWORDS

high blood sugar – non-enzymatic glycation – *in vitro* protocol – glucose incubation – erythrocyte glycation

1. BACKGROUND

1.1 INTRODUCTION

Glycation is a term that refers to spontaneous, non-enzymatic, and slow reactions between the amino groups of proteins and the carbonyl group of a reduction sugar, like glucose (Bucala and Cerami, 1992). Initially, it is a reversible reaction, and eventually, the newly formed structure rearranges into a more stable conformation. This series of complex transformations lead to the formation of compounds called advanced glycation end-products (AGE) (Ahmed and Thornalley, 2007; Yuan *et al.*, 2011; Elaheh and Abdolhassan, 2018; Dariya and Nagaraju, 2020).

In pathological conditions, such as diabetes, where high glucose levels in the blood are sustained for a long time, the cellular and extracellular proteins can suffer irreversible damage because of the glycation process (Vlassara and Palace, 2002; Guo *et al.*, 2009; Broz *et al.*, 2021). However, study these changes in samples from diabetic patients is complicated because of the comorbidities related to diabetes (hypertension, obesity, hyperlipidemia, etc.) that can influence the results. For this reason, several researchers have developed *in vitro* protocols for study the glycation process.

The *in vitro* glycation of red blood cells (RBCs) differs from *in vivo* glycation, particularly in the physiological changes, glucose metabolism, and continuous renewal of RBCs in the blood flow. Nonetheless, it is useful to study specifically the glucose effects on the RBCs in media at different glucose concentrations. Several researchers have proposed different protocols for *in vitro* controlled glycation, where the RBCs alterations were evaluated with different experimental techniques (Raftos *et al.*, 2001; Nagai *et al.*, 2005; Resmi *et al.*, 2005; Riquelme *et al.*, 2005; Selvaraj *et al.*, 2006; Nam *et al.*, 2009; Lemos *et al.*, 2011; Viskupicova *et al.*, 2011; Korol *et al.*, 2011 and 2013; Sompong *et al.*, 2015; Buszniez *et al.*, 2019; Catan *et al.*, 2019; Turpin *et al.*, 2020; Batista da Silva *et al.*, 2021). They employed different levels of glucose concentration, and incubation times varying from minutes to days. In these tests, the glycation level and biochemical and hemorheological changes of the RBCs incubated with glucose solution under different conditions were analyzed.

In the present methods article, some of the most relevant protocols found in the literature for *in vitro* glycation of RBC were analyzed to compare them with each other. Through this analysis, an optimal protocol for *in vitro* glycation of RBCs was proposed.

1.2 PROTOCOLS REVISION

The available literature was analyzed to compare the values of each incubation condition used by each author. The incubation parameters used for *in vitro* glycation were organized and presented in Table 1. Some of the analyzed bibliographies use different units to express the glucose concentration. Therefore, to compare the protocols, the notation in Table 1 was standardized to show the glucose values in g/dl.

Suspension medium

The suspension medium is chosen to simulate the conditions *in vivo* so that cells can live. Therefore, the suspension medium used during the incubation has a fundamental role in the *in vitro* assays. As reported in the analyzed literature, the most commonly used suspension medium was phosphate-buffered saline (PBS) with the same pH and osmolality as the human plasma (pH 7.4 and 300 mOsM). Table 1 shows the different suspension media used in the consulted bibliography.

Hematocrit

The ratio of the volume of RBCs to the total volume of blood is called hematocrit (HCT). To simulate *in vitro* what occurs *in vivo*, the incubation concentration of RBCs in the medium should be within the normal HCT range (between 38% and 42%) (Guyton and Hall, 2011). Most of the HCT values found in the analyzed literature vary between 38% and 50%. In general, the HCT of these protocols did not exceed the value of 50%. However, it is important to mention that Catan *et al.* (2019), Lemos *et al.* (2011), Nagai *et al.* (2005), Nam *et al.* (2009), Raftos *et al.* (2001), Selvaraj *et al.* (2006), Sompong *et al.* (2015), Turpin *et al.* (2020), and Viskupicova *et al.* (2011) used a HCT lower than the normal value for their tests. On the other hand, the concentrations used by Korol *et al.* (2013) and Resmi *et al.* (2005) were within the normal range. Furthermore, the researchers Batista da Silva *et al.* (2021), Buszniez *et al.* (2019), Korol *et al.* (2011), and Riquelme *et al.* (2005), used 50%, slightly above the normal HCT.

Instead of HCT, RBCs count (number of cells per unit of volume) is more appropriate for several analyses. For these cases, it is usually considered the value (4.7 ± 0.3) 10^6 RBC/mm^3, corresponding to the RBCs count estimated for a healthy woman (Guyton and Hall, 2011).

Glucose concentration

According to standard biochemical analysis, the normal fasting (no food for eight hours) blood sugar level is between 0.070 g/dl and 0.099 g/dl. Prediabetes is diagnosed between 0.100 g/dl and 0.125 g/dl, and diabetes is diagnosed when the fasting blood glucose is equal to or greater than 0.126 g/dl (Guyton and Hall, 2011; Alvin, 2016; Centers for disease control and prevention, 2021). The values of glucose concentration during the incubation vary between 0.09 g/dl and 6 g/dl in all analyzed protocols (Table 1). These glucose concentrations simulate the RBCs alterations by hyperglycemia in diabetes patients (Foresto *et al.*, 2000; Delannoy *et al.*, 2014; Alet *et al.*, 2016).

Glucose available per unit volume of RBCs

The amount of glucose available during the incubation for a volume of RBCs is obtained considering the glucose concentration and the HCT used by each researcher. Lemos *et al.* (2011) and Nam *et al.* (2009) employed the highest glucose amount for incubation, while Buszniez *et al.* (2019), Resmi *et al.* (2005), and Riquelme *et al.* (2005) used the lowest glucose amount for incubation. The other protocols used different concentrations of glucose, which were between 0.4 g/dl and 18 g/dl of RBCs.

Estimated amount of glucose per RBC

The analysis of the amount of glucose for each RBC during the incubation showed that Lemos *et al.* (2011), Nam *et al.* (2009), Raftos *et al.* (2001), Viskupicova *et al.* (2011), Turpin *et al.* (2020), Catan *et al.* (2019), Sompong *et al.* (2015), and Nagai *et al.* (2005) used the highest amount of glucose per RBC (between 3,191 10^{-12} g and 11.5 10^{-12} g). In

contrast, the other protocols used amounts of glucose that vary between 5.3 10^{-12} g and 0.4 10^{-12} g, presenting an average value of 2.1 10^{-12} g per RBC.

Incubation time

It has been shown that *in vitro* glycation can be affected by both incubation time and glucose concentration (Nagai *et al.*, 2005; Sompong *et al.*, 2015). In the analyzed literature, incubation times vary from 0.5 to 120 hours. Fig. 1 shows the incubation times and the glucose concentrations used in the analyzed protocols, showing that the glucose concentration is below 6 g/dl for 60% of the protocols. Furthermore, the incubation times are long in some tests (Raftos *et al.*, 2001; Nagai *et al.*, 2005; Resmi *et al.*, 2005; Selvaraj *et al.*, 2006; Viskupicova *et al.*, 2011; Sompong *et al.*, 2015; Catan *et al.*, 2019; Turpin *et al.*, 2020). These longtime could be troublesome because glucose concentration decreases due to cellular metabolism. On the other hand, some assays whose incubation times were shorter used higher concentrations of glucose (Nam *et al.*, 2009; Lemos *et al.*, 2011). However, most of the tests analyzed used a wide range of glucose concentration (Table 1).

Temperature

Temperature is a very important factor for the incubation of cells. In the analyzed literature, all authors used the temperature of 37 °C which is the average body temperature (Yuan *et al.*, 2011) (values not shown in Table 1).

Techniques of analysis

Lemos *et al.* (2011) employed the spectrophotometry technique measuring the absorbance of the supernatants (after hemolysis) to determine the erythrocyte stability against glucose and hypotonic stress, the influence of NaCl on the hemoglobin, and the hemoglobin stability against glucose. Raftos *et al.* (2001) used the same technique to obtain the hemoglobin concentration as cyanmethemoglobin. The spectrophotometry technique was also used by: Viskupicova *et al.* (2011) to measure the hemoglobin and

glycated hemoglobin concentration; Resmi *et al.* (2005) to determine hemoglobin levels. Also, Turpin *et al.* (2020) used turbidimetry (a type of spectrophotometry technique) to measure the capacity of erythrocyte to resist lysis induced by oxidative stress, and Catan *et al.* (2019) to measure the hemolysis degree. Catan *et al.* (2019) used HPLC to measure the glycated hemoglobin.

Nagai *et al.* (2005) used gas chromatography with mass spectrometry to measure AGE concentration. Selvaraj *et al.* (2006) employed the same instrument to measure the glycated hemoglobin concentration, the influence by malondialdehyde (MDA) on hemoglobin, and MDA concentration. Turpin *et al.* (2020) used Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) to determine the glycation of α and β hemoglobin.

The flow cytometer technique was used by: Catan *et al.* (2019) to analyze conformational changes, glycation effects, and aging in erythrocytes; Sompong *et al.* (2015) to measure phosphatidylserine exposure, glucose concentration, and glycated hemoglobin; Turpin *et al.* (2020) to determine the erythrocyte shape, eryptosis and intracellular reactive oxygen species; and Viskupicova *et al.* (2011) to measure intracellular superoxide and to evaluate the eryptosis.

Buszniez *et al.* (2019) employed a glucometer to measure glucose concentration during the incubation, while Raftos *et al.* (2001) used the Trinder glucose activity test, and Resmi *et al.* (2005) used an autoanalyzer.

Turpin *et al.* (2020) and Nam *et al.* (2009) used an ektacytometry technique to determine the erythrocyte membrane deformability. Riquelme *et al.* (2005) used an erythrocyte rheometer (Riquelme *et al.*, 2013; Riquelme *et al.*, 2018) and digital image analysis to analyze the alterations on viscoelasticity (Riquelme *et al.*, 1998; Riquelme *et al.*, 2000a; Riquelme *et al.*, 2000b) and aggregation of RBCs incubated with glucose solutions (Alet

et al., 2012). Also, Korol *et al.* (2011 and 2013) used the erythrocyte rheometer to analyze the nonlinear disorder in the erythrocyte viscoelasticity. Furthermore, Batista da Silva *et al.* (2021) employed the same techniques that Riquelme *et al.* (2005), also incorporating an optical chip aggregometer based on light transmission (Toderi *et al.*, 2015; Toderi *et al.,* 2017) to analyze the erythrocyte aggregation kinetics. Nam *et al.* (2009) employed a microchip-stirring system to quantify erythrocyte aggregation. Resmi *et al.* (2005) used the CTA cell counts to measure and evaluate RBCs deformability.

2. METHODS

The analyzed bibliography employed different parameters to evaluate the *in vitro* glycation of RBCs. Therefore, and considering our previous assays, we propose a new protocol, which was validated as depicted in the following sections.

2.1 BLOOD SAMPLES

Human blood samples from two healthy male donors (27 years old, no intake of any medication for the last 7 days, non-smoker, normotensive, with normal hematological values and without any pre-existing health problems) were collected by venipuncture and anticoagulated with EDTA-K_2. Collection and processing of samples were performed within 4 h from extraction (Baskurt *et al.*, 2007). The study was approved by the Bioethics Committee of the Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR (Res. N°347/2013) and the donors signed the informed consent. Plasma was separated by gentle centrifugation (1.500g, 5 min at room temperature). Then, RBCs were washed twice with PBS (pH 7.4 and 300 mOsM).

2.2 RBCs GLYCATION

The incubation medium was prepared solving 0.04 g of glucose (dextrose, Biopack, batch 16882015) in 0.02 dl of PBS with human serum albumin 0.5% v/v (human serum albumin 20% UNC, batch AH2003/50-20). The samples were prepared by adding one volume of washed RBC and one volume of the incubation medium. Then, the final concentration of glucose was 1 g/dl, and the final HCT was 50%.

The incubation was carried out for 5 hours at 36.5 ºC under constant stirring. After incubation, the RBCs were washed twice with PBS by gentle centrifugation (1.500g, 5 min at room temperature).

2.3 ANALYSIS OF RBC ALTERATION

Erythrocyte viscoelasticity

The erythrocyte rheometer (Riquelme *et al.*, 2013; Riquelme *et al.*, 2018) was used to determine the following erythrocyte viscoelastic parameters (Riquelme *et al.*, 1998; Riquelme *et al.*, 2000a; Riquelme *et al.*, 2000b):

μ: Elastic modulus

η_m: Membrane surface viscosity

G': Dynamic elastic modulus

G": Viscous modulus

η': Viscous component of the dynamics viscosity

η": Elastic component of the dynamics viscosity

The dynamics viscoelastic parameters G', G", η', and η" were determined at 1 Hz of oscillatory frequency (equivalent to a cardiac frequency of 60 cycles per minute). Briefly, the RBCs (Control or Glucose-treated) were suspended at 40% HCT in autologous plasma. Then, 110 μl of these suspensions were poured in 4.5 ml of a solution of

polyvinyl-pyrrolidone (PVP360®, Sigma) at 5% (w/v) in PBS (pH 7.4, 300 mOsm/kg, and 22 cP at 25 °C).

Erythrocyte aggregation

The optical chip aggregometer (Toderi *et al.*, 2015; Toderi *et al.,* 2017) was used to obtain the following aggregation kinetics parameters of RBCs:

$Amp_{1/2}$: The half of the difference between minimum and maximum of light intensity in the syllectrogram.

$t_{1/2}$: The time required to reach the $Amp_{1/2}$, indicating the characteristic time constant for the aggregation.

The study of the RBC aggregation was performed by analyzing the digital images obtained by microscopy (Union Tokyo Optical, 40x objective). Briefly, the RBCs were suspended at 0.003 % in autologous plasma, and then 20 µl of each suspension were placed on a slide for observation. After 5 min, six images were recorded using an attached digital camera (Mikoba 300 CMOS 3.0). The images were visualized using the software ISCapture, and the aggregates were categorized according to their number of RBC. Subsequently, the percentages corresponding to each category (Danieli *et al.*, 2009) were calculated and expressed as:

% individual RBC

% aggregates 2, 3, 4 RBCs

% aggregates ≥ 5 RBCs

% large aggregate networks

Glycated hemoglobin

The glycated hemoglobin (HbA1c) was determined by the immunoturbidimetric technique employing a commercial kit (GTlab) and a biochemistry analyzer (A25, Biosystems).

Statistical Analysis

Data were expressed as mean ± standard deviation. The unpaired two-tailed Student t-test was performed with InfoStat (Di Rienzo *et al.*, 2018). Each parameter was grouped by each assay, and the differences were considered significant at $p<0.05$.

2.4 RESULTS AND DISCUSSION

Fig. 2 shows representative images of RBCs incubated in PBS (Control), and in the solution of 1 g/dl glucose (Glucose-treated). Table 2 shows the parameters obtained by erythrocyte rheometer, optical chip aggregometer and digital image analyzes, and the levels of HbA1c.

Table 2 shows significant differences of μ and η_m from Glucose-treated RBCs ($p<0.05$), indicating changes in the erythrocyte membrane. The viscous modulus (G") and the dynamic viscosity (η') showed a significant increase in the Glucose-treated RBCs ($p<0.05$). These results show an interaction of glucose with the lipid bilayer and slight changes in the erythrocyte elasticity. Moreover, the rheological parameter alterations were similar to those found in RBCs from diabetic donors (Torregiani *et al.*, 1995).

The aggregation kinetics analysis showed that the $Amp_{1/2}$ of Glucose-treated RBCs was significantly increased and $t_{1/2}$ decreased ($p<0.05$), meaning that Glucose-treated RBCs could aggregate faster than Control. Also, the analysis of RBCs aggregate images indicated a significant decrease in the percentage of individual cells and the aggregates of 2, 3, or 4 cells for the Glucose-treated RBCs ($p<0.0005$ and $p<0.05$, respectively). Furthermore, RBC aggregates of 5 or more cells and large aggregate networks presented a significant increase suggesting that glucose could facilitate the formation of large aggregates ($p<0.005$). These results indicate that the Glucose-treatment would lead to a decrease of the electrostatic repulsion between cells. Consequently, this treatment

increased erythrocyte aggregation, similar to the reported in diabetic patients (Delannoy *et al.*, 2015).

These findings indicate that this *in vitro* Glucose-treatment could simulate the effects of hyperglycemia in diabetes. Moreover, Table 2 shows that the Glucose-treated RBCs presented a significantly higher HbAc1 when compared to Control ($p<0.005$). Although the values are lower than those corresponding to diabetic patients (Centers for disease control and prevention, 2021), our results indicate that the proposed protocol would produce similar effects on erythrocytes as hyperglycemia in diabetes.

2.5 PROPOSED PROTOCOL

The glucose concentration for incubation can be variable, depending on the aim of the study. However, glucose values must be higher than those found under physiological *in vivo* conditions to achieve the glycation effect *in vitro*. We suggest 1g/dl final concentration as a minimum value to achieve a detectable difference for the hemorheological parameters between control and treated samples, similar to those found in diabetes (Torregiani *et al.*, 1995).

The HCT during the incubation should be as close to the physiological values as possible. Then, 50% HCT is the most appropriated since it is an average between the previously mentioned ranges (Guyton and Hall, 2011).

Considering the estimated amount of RBC from a healthy woman (Guyton and Hall, 2011), the expected glucose value for each RBC during incubation would be $4.2\ 10^{-12}$ g approximately.

The incubation media and time can also be variable according to the study aim. It is relevant to clarify that for long incubations (120 and 48 hours), some authors employed non-physiological compounds such as the DEDM (Nagai *et al.*, 2005), PBS with pen-

strept (Resmi *et al.*, 2005; Selvaraj *et al.*, 2006), and PBS with ampicillin (Viskupicova *et al.*, 2011) to avoid contamination. However, this non-physiological media could interfere with the correct interpretation of the glycation phenomenon. For this reason, we recommend incubation in PBSA media at 36.5°C for no more than 5 hours to avoid contamination.

Finally, considering all the data analyzed, an RBC glycation protocol is suggested to perform a suitable *in vitro* incubation, following these steps:

1) Obtain human blood samples by venipuncture from male donors and anticoagulate with EDTA-K_2. Separate the plasma by gentle centrifugation (1.500g, 5 min at room temperature) and remove the buffy coat. Wash the RBCs twice with PBS (pH 7.4, 300 mOsM) and discard the supernatant.

2) Prepare the PBSA solution by adding human serum albumin (final concentration of 1% v/v) to PBS (pH 7.4, 300 mOsM), and the Glucose solution supplementing PBSA with dextrose (final concentration 2% v/v).

3) Incubate 1 volume of glucose solution with 1 volume of washed RBCs. Carry out the incubation for 5 hours at 36.5 °C under constant stirring. After incubation, wash the RBCs twice with PBS and reserve the erythrocytes for further determinations.

3. APPLICATIONS

The main application for the proposed protocol is the analysis of the erythrocyte glycation consequence in diabetes without the influence of the related comorbidities (Torregiani *et al.*, 1995; Delannoy *et al.*, 2015; Alet *et al.*, 2016). Another important application is the pharmacologic evaluation of diabetes treatment (Mascaro Grosso *et al.*, 2017). Also, it is suitable to analyze the effect of glucose on RBCs in different suspension media, such as the ones employed in the erythrocyte storage (Foresto *et al.*, 2000).

Moreover, this protocol could be used to the *in vitro* evaluation of the effect that different agents would have in diabetic patients. For example, the effect on glycated RBCs of different anesthetics (Alet *et al.*, 2021; Batista da Silva *et al.*, 2021) and phytochemical compounds (Buszniez *et al.*, 2017; Riquelme *et al.*, 2019) can be studied employing this glycation protocol.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

ACKNOWLEDGEMENTS

The authors would like to extend our gratitude to Universidad Nacional de Rosario, and CONICET for giving access to the bibliography consulted. Also, the authors are grateful to Dr. Germán Detarsio of the Department of Hematology (FCByF, UNR) for the glycated hemoglobin determinations.

GRANTS

This work was supported by the Universidad Nacional de Rosario (grant number BIO400, Res. CS. N° 448/2017). Marcus V. Batista da Silva is a doctoral fellow from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina).

REFERENCES

Ahmed N., Thornalley P., 2007. Advanced glycation endproducts: what is their relevance to diabetic complications? Diabetes Obes. Metab. 9, 233-245. DOI: 10.1111/j.1463-1326.2006.00595.x

Alet A., Alet N., Delannoy M., Fontana A., Riquelme B., 2012. *In vitro* study of rheological effects of propofol on human erythrocyte membrane. Ser. Biomech. 27, 39-44

Alet A., Basso S., Castellini H., Delannoy M., Alet N., D'Arrigo M., Riquelme B., 2016. Hemorheological *in vitro* action of propofol on erythrocytes from healthy donors and diabetic patients. Clin. Hemorheol. Microcirc. 64, 157-165. DOI: 10.3233/CH-162054

Alet A., Batista da Silva M., Castellini H., Alet N., Riquelme B., 2021. *In vitro* alteration on erythrocytes mechanical properties by propofol, remifentanil and vecuronium. Microvasc. Res. 135, 104132. DOI:10.1016/j.mvr.2021.104132

Alvin CP., 2016. Diabetes mellitus: diagnóstico, clasificación y fisiopatología, in Harrison. Principios de Medicina Interna (Kasper, D, Fauci, A, Hauser, S, Longo, D, Jameson, JL, Loscalzo, J ed), 19 ed. McGraw-Hill Interamericana Editores, S.A., chap. 417

Baskurt OK., Hardeman MR., Rampling MW., Meiselman HJ., 2007. Handbook of Hemorheology and Hemodynamics. 1st ed. IOS Press, Amsterdam

Batista da Silva M., Castellini H., Alet N., Riquelme B., Alet A., 2021. A preliminary study of the effect of anesthetic agents on the rheological parameters of glycated erythrocytes. Anales. AFA. 31, 117-120. DOI: 10.31527/analesafa.2020.31.4.117

Broz K., Walk R., Tang S., 2021. Complications in the spine associated with type 2 diabetes: the role of advanced glycation end-products. Med. Nov. Technol. Devices. 11, 100065. DOI: 10.1016/j.medntd.2021.100065

Bucala R., Cerami A., 1992. Advanced glycosylation: chemistry, biology, and implications for diabetes and aging. Adv. Pharmacol. 23, 1-34. DOI: 10.1016/s1054-3589(08)60961-8

Buszniez P., Lerda N., D'Arrigo M., Riquelme B., 2017. Hemorheological action of trigonelline on *in vitro* glycated red blood cells. Ser. Biomech. 31, 30-35

Buszniez P., Mascaro Grosso H., Pusillico R., D'Arrigo M., Riquelme B., 2019. Relationship between glucose consumption and alteration in erythrocyte aggregation in a non-enzymatic glycosylation model *in vitro*. Biocell. 43 (Suppl. 2), A13. https://www.techscience.com/biocell/v43nSuppl.2/33860

Catan A., Turpin C., Diotel N., Patche J., Guerin-Dubourg A., Debussche X., Bourdon E., Ah-You N., Le Moullec N., Besnard M., Veerapen R., Rondeau P., Meilhac O., 2019. Aging and glycation promote erythrocyte phagocytosis by human endothelial cells: Potential impact in atherothrombosis under diabetic conditions. Atherosclerosis. 291, 87-98. DOI: 10.1016/j.atherosclerosis.2019.10.015

Centers for disease control and prevention. "Diabetes Tests". URL: https://www.cdc.gov/diabetes/basics/getting-tested.html#:~:text=A%20fasting%20blood%20sugar%20level,higher%20indicates%20you%20have%20diabetes [Accessed May 2021]

Danieli V., Fontana A., Alessi A., Foresto P., Valverde J., Grandfils C., Riquelme B., 2009. Tratamiento de eritrocitos humanos con copolímeros sintéticos: optimización del tiempo de incubación. Acta Bioquim. Clin. Latinoam. 43 (1): 43-48 ISSN: 0325-2957

Dariya B., Nagaraju G., 2020. Advanced glycation end products in diabetes, cancer and phytochemical therapy. Drug. Discov. Today. 25, 1614-1623. DOI: 10.1016/j.drudis.2020.07.003

Delannoy M., Fontana A., D'Arrigo M., Riquelme B., 2015. Influence of hypertension and diabetes on erythrocyte aggregation using image digital analysis. Ser. Biomech. 29, 5-10

Di Rienzo J., Casanoves F., Balzarini M., Gonzalez L., Tablada M., Robledo C., 2018. InfoStat, Version 2018. Centro de Transferencia InfoStat, FCA, Universidad Nacional de Córdoba, Argentina

Elaheh F., Abdolhassan K., 2018. Review on Advanced Glycation End Products and the Progress of Chronic Disorders. Curr. Trends. Biomedical. Eng. & Biosci. 12, 91-92. DOI:10.19080/CTBEB.2018.12.555846

Foresto P., D'Arrigo M., Carreras L., Cuezzo RE., Valverde J., Rasia RJ., 2000. Evaluation of red blood cell aggregation in diabetes by computerized image analysis. Medicina. 60, 570-572. PMID: 11188894

Guo X., Wang L., Chen B., Li Q., Wang J., Zhao M., Wu W., Zhu P., Huang X., Huang Q., 2009. ERM protein moesin is phosphorylated by advanced glycation end products and modulates endothelial permeability. Am. J. Physiol. Heart. Circ. Physiol. 297, H238-H246. DOI: 10.1152/ajpheart.00196.2009

Guyton AC., Hall JE., 2011. Tratado de Fisiologia Médica, 12ed. Brazil, Elsevier

Korol A., Rosso O., Martin MT., D'Arrigo M., Riquelme B., 2011. Impairment of erythrocytes incubated in glucose medium: A Wavelet-Information Theory Analysis. Cell. Biochem. Biophys. 60, 329-334. DOI: 10.1007/s12013-011-9155-y

Korol A., Riquelme B., D'Arrigo M., 2013. Crossover from Weak to Strong Nonlinear Disorder in the Viscoelasticity of Glucose Incubated Erythrocytes. Open J. Biophys. 3, 191-197. DOI: 10.4236/ojbiphy.2013.33022

Lemos G., Márquez-Bernardes L., Arvelos L., Paraíso L., Penha-Silva N., 2011. Influence of Glucose Concentration on the Membrane Stability of Human Erythrocytes. Cell. Biochem. Biophys. 61, 531–537. DOI: 10.1007/s12013-011-9235-z

Mascaro Grosso H., Buszniez P., Estrada E., Riquelme B., 2017. Estudio comparativo de los parámetros reológicos, ópticos y fisicoquímicos de extractos acuosos de hojas de Bauhinia forficata. Ars Pharm. 58(2), 83-85. https://revistaseug.ugr.es/index.php/ars/article/view/6383

Nagai R., Deemer E., Brock J., Thorpe S., Baynes J., 2005. Effect of glucose concentration on formation of AGEs in erythrocytes *in vitro*. Ann. N. Y. Acad. Sci. 1043, 146-150. DOI: 10.1196/annals.1333.018

Nam J., Kim C., Shin S., 2009. The effect of L-carnosine on the rheological characteristics of erythrocytes incubated in glucose media. Korea-Aust. Rheol. J. 21,103-108

Raftos J., Edgley A., Bookchin R., Etzion Z., Lew V., Tiffert T., 2001. Normal Ca^{2+} extrusion by the Ca^{2+} pump of intact red blood cells exposed to high glucose concentrations. Am. J. Physiol. Cell Physiol. 280, C1449-C1454. DOI: 10.1152/ajpcell.2001.280.6.C1449

Resmi H., Akhunlar H., Artmann AT., Güner G., 2005. *In vitro* effects of high glucose concentrations on membrane protein oxidation, G-actin and deformability of human erythrocytes. Cell Biochem. Funct. 23, 163-168. DOI: 10.1002/cbf.1129

Riquelme B., Valverde J., Rasia RJ., 1998. Complex viscoelasticity of normal and lectin treated erythrocytes using laser diffractometry. Biorheology. 35, 325-334. DOI: 10.1016/S0006-355X(99)80014-6

Riquelme B., Valverde J., Rasia RJ., 2000a. Determination of the complex viscoelastic parameters of human red blood cells by laser diffractometry. Optical Diagnostics of Biological Fluids V. 3923, 132-140. DOI:10.1117/12.387133

Riquelme B., Foresto P., Valverde J., Rasia RJ., 2000b. Alterations to complex viscoelasticity of erythrocytes during storage. Clin. Hemorheol. Microcirc. 22, 181-188. PMID: 10976711

Riquelme B., Foresto P., D'Arrigo M., Valverde J., Rasia RJ., 2005. A dynamic and stationary rheological study of erythrocytes incubated in a glucose medium. J. Biochem. Biophys. Methods. 62, 131-141. DOI: 10.1016/j.jbbm.2004.10.004

Riquelme B., Albea B., Marenzana A., Castellini H., 2013. Reómetro Eritrocitario. Invention Patent AR 091467 B1. https://lens.org/002-392-548-778-992

Riquelme B., Castellini H., Albea B., 2018. Linear and Nonlinear Viscoelasticity of Red Blood Cells using a New Optical Erythrocyte Rheometer. OSA Technical Digest. Th4A.41. https://doi.org/10.1364/LAOP.2018.Th4A.41

Riquelme B., Grosso H., Toderi M., Buszniez P., D'Arrigo M., 2019. *In vitro* effect of trigonelline on the erythrocyte aggregation in a model of diabetes. Anais do Encontro Anual da Biofísica, Blucher Biophysics. 2, 77-78. DOI: 10.5151/biofisica2019-22

Selvaraj N., Zachariah B., Sathiyapriya V., 2006. Effect of lipid peroxides and antioxidants on glycation of hemoglobin: An in vitro study on human erythrocytes. Clin. Chim. Acta. 366, 190-195. DOI:10.1016/j.cca.2005.10.002

Sompong W., Cheng H., Adisakwattana S., 2015. Protective Effects of Ferulic Acid on High Glucose-Induced Protein Glycation, Lipid Peroxidation, and Membrane Ion Pump Activity in Human Erythrocytes. PLoS One. 10 (6), e0129495.

DOI:10.1371/journal.pone.0129495

Toderi M., Castellini H., Riquelme B., 2015. Simplified variant of an optical chip to evaluate aggregation of red blood cells. Biophotonics South America, Proc. SPIE. 9531, 95313X. DOI: 10.1117/12.2180907

Toderi M., Castellini H., Riquelme B., 2017. Descriptive parameters of the erythrocyte aggregation phenomenon using a laser transmission optical chip. J. Biomed. Opt. 22, 017003. DOI: 10.1117/1.JBO.22.1.017003

Torregiani F., Umansky M., Riquelme B., Rasia RJ., 1995. Hemorheologic Alterations in diabetic patients. Clin. Hemorheol. Microcirc. 15, 687-690

Turpin C., Catan A., Guerin-Dubourg A., Debussche X., Bravo SB., Álvarez E., Van Den Elsen J., Meilhac O., Rondeau P., Bourdon E., 2020. Enhanced oxidative stress and damage in glycated erythrocytes. PLoS One. 15 (7), e0235335. https://doi.org/10.1371/journal.pone.0235335

Viskupicova J., Blaskovic D., Galiniak S., Soszyński M., Bartosz G., Horakova L., Sadowska-Bartosz I., 2011. Effect of high glucose concentrations on human erythrocytes *in vitro*. Redox Biol. 5, 381-387. DOI: 10.1016/j.redox.2015.06.011

Vlassara H., Palace M., 2002. Diabetes and advanced glycation end products. J. Intern. Med. 251, 87-101. DOI: 10.1046/j.1365-2796.2002.00932.x

Yuan Y., Zhao L., Chen Y., Moorhead J., Varghese Z., Powis S., Minogue S., Sun Z., Ruan X., 2011. Advanced glycation end products (AGEs) increase human mesangial foam cell formation by increasing Golgi SCAP glycosylation *in vitro*. Am. J. Physiol. Renal. Physiol. 301, F236-F243. DOI: 10.1152/ajprenal.00646.2010

FIGURE LEGENDS

Fig. 1. Glucose per unit volume of RBC as a function of incubation time (logarithmic scale).

Fig. 2. Representative images obtained from RBCs incubated in PBS (Control), and in the solution of 1g/dl glucose (Glucose-treated).

TABLE LEGENDS

Table 1. *Comparative analysis of glucose incubation conditions*

Table 2. *Parameters of Control and Glucose-treated RBC*

Table 1. *Comparative analysis of glucose incubation conditions*

Ref*	Author	Suspension medium	HCT (%)	Glucose concentration (g/dl)	Glucose per unit volume of RBC (g/dl)	Glucose per RBC** (10^{-12} g)	Incubation time (hours)
P1	Batista da Silva *et al.* (2021)	PBS (137 mM NaCl, 1.4 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2.7 mM KCl)	50	0.25	0.5	1.06	5
P2	Buszniez *et al.* (2019)	PBS (137 mM NaCl, 1.4 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2.7 mM KCl)	50	0.1; 0.2; 0.3; 0.4; and 0.5	0.2; 0.4; 0.6; 0.8 and 1	0.4; 0.9; 1.3; 1.7; and 2.1	4
P3	Catan *et al.* (2019)	Phosphate Buffered Saline solution (1X PBS)	20	0.09; 0.31; 0.54; 0.99; 1.89; and 2.34	0.45; 1.55; 2.7; 4.95; 9.45; and 11.7	0.96; 3.29; 5.74; 10.5; 20.1; and 24.9	120
P4	Korol *et al.* (2011)	PBS (137 mM NaCl, 1.4 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2.7 mM KCl)	50	0.2; 0.5; and 1	0.4; 1; and 2	0.9; 2.1 and 4.2	2
P5	Korol *et al.* (2013)	PBS (137 mM NaCl, 1.4 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2.7 mM KCl)	40	0.2; 0.5; and 1	0.5; 1.25; and 2.5	1.06; 2.6; and 5.3	2
P6	Lemos *et al.* (2011)	Solution with or without NaCl (0.1 – 1.0 g/dl)	0.4	0.1; 0.3; 0.6; 1.2; 2; 4; and 6.0	25; 75; 150; 300; 500; 1000; and 1500	53.2; 159; 319; 638; 1064; 212; and 3191	0.5
P7	Nagai *et al.* (2005)	cell culture media (DMEM, Dulbecco's modified Eagle's medium)	10	0.09 and 0.54	0.9 and 5.4	1.9 and 11.5	120
P8	Nam *et. al.* (2009)	solution with NaCl (0.9 g/dl)	10	2.4; 2.6; and 2.8	24; 26; and 28	51.0; 55.3; and 59.5	0.5
P9	Raftos *et al.* (2001)	Solution A: 80 mM KCl, 70 mM NaCl, 0.15 mM $MgCl_2$, 10.0 mM HEPES-sodium (pH 7.55 at 37°C), and 0.1 mM EDTA-Na_2 Solution B: prepared like "solution A" without EDTA-Na_2	10	1st test: 0.18 to 0.54 2nd test: 1.8	1er test: 1.8 to 5.4 2do test: 18	1er test: 3.8 to 11.5 2do test: 38.3	1er test: 3 2do test: 6
P10	Resmi *et al.* (2005)	PBS (5mM NaH_2PO_4 plus 138mM NaCl) supplemented with pen-strept	38	0.09; 0.18; 0.45; and 0.81	0.2; 0.5; 1.2; and 2	0.5; 1; 2.5; and 4.5	48
P11	Riquelme *et al.* (2005)	PBS (137 mM NaCl, 1.4 mM KH_2PO_4, 10 mM Na_2HPO_4, and 2.7 mM KCl)	50	0.1; 0.2; 0.5; and 1	0.2; 0.4; 1; and 2	0.4; 0.9; 2.1; and 4.2	2

P12	Selvaraj *et. al.* (2006)	PBS (140 mM NaCl, 16 mM Na_2HPO_4, 1.0 mM NaH_2PO_4) supplemented with penicillin and streptomycin.	10	0.09	0.9	1.91	24
P13	Sompong *et al.* (2015)	PBS (140 mM NaCl, 1.0 mM NaH_2PO_4,16 mM Na_2HPO_4)	10	0.09; 0.27; 0.54; and 0.81	0.9; 2.7; 5.4; and 8.1	1.91; 5.74; 11.5; and 17.2	24
P14	Turpin *et al.* (2020)	PBS (not specified)	20	0.09; 0.45; and 2.5	0.45; 2.25; and 12.2	0.95; 4.78; and 26.6	120
P15	Viskupicova *et al.* (2011)	PBS: 1 tablet of PBS/100 ml H_2O (140 mM NaCl, 10 mM NaH_2PO_4, 3 mM KCl) supplemented with ampicillin.	10	0.09; 0.81; and 1.8	0.9; 8.1; and 18	1.91; 17.2; and 38.3	24, 48; and 72

*Reference code; **Using the estimated value for healthy woman, $(4.7 \pm 0.3)\ 10^6$ RBC/mm³ (Guyton and Hall, 2011)

Table 2. *Parameters of Control and Glucose-treated RBC*

Parameters	**Control**	**Glucose-treated**
μ [10^{-6} N/m]	5.11 ± 0.02	5.19 ± 0.04*
η_m [10^{-7} N.s/m]	1.9 ± 0.4	2.19 ± 0.08*
G' [10^{-6} N/m]	4.97 ± 0.03	4.99 ± 0.05
G" [10^{-6} N/m]	1.2 ± 0.1	1.6 ± 0.2*
η' [10^{-7} N.s/m]	3.9 ± 0.4	5.1 ± 0.3*
η'' [10^{-6} N.s/m]	1.58 ± 0.02	1.56 ± 0.05
$Amp_{1/2}$ [A. U.]	89.3 ± 0.8	94.4 ± 0.2*
$t_{1/2}$ [s]	24.5 ± 0.5	18.0 ± 0.1*
% Individual RBC	29 ± 1	13 ± 1***
% Aggregates 2, 3, 4 RBCs	51 ± 7	20 ± 6*
% Aggregates ≥ 5 RBCs	17 ± 2	56 ± 8**
% Large aggregate networks	3 ± 1	11 ± 2**
HbA1c [%]	2.70 ± 0.05	3.10 ± 0.06**

*p<0.05; **p<0.005; ***p<0.0005; [A. U.] Arbitrary Units