

## A Simple Method to Quantify Glycogen from Human Platelets

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### Abstract

**Objective:** The aim of our study was to establish a protocol to quantify the glycogen from human platelets. **Methods:** The total glycogen was determined in the platelet concentrate (PC) obtained from healthy male volunteers. The assay comprised the following steps: (1) cell lysing and glycogen extraction, (2) precipitation of glycogen and its washing, (3) conversion of glycogen to free glucose, (4) neutralization, and (5) detection of free glucose. **Results:** In our analysis, the measured glycogen content in the PC was  $0.263 \pm 0.101 \mu\text{g}/10^6$  platelets (mean  $\pm$  SD,  $n=16$ ). **Discussion:** Despite the potential importance of glycogen determination in the platelets, a biochemical method to quantify this polymer in these cells is not available at the moment. Our protocol proved to be a simple method to measure these reserves from a blood sample. We expect to contribute to future studies of platelet metabolism, especially regarding glycogen function in these cells.

**Keywords:** Alzheimer's Disease; GSK3 $\beta$ ; Depression; Diabetes

**Abbreviations:** ADP: D-Hexose-6-Phosphotransferase; GS: Glycogen Synthase; GP: Glycogen Phosphorilase; PRP: Platelet-Rich Plasma; PC: Platelet Concentrate; G: Glycogen; G<sub>s</sub>: Glycogen Concentration Standard; Abs<sub>s</sub>: Absorbance of Glucose Standard; Abs<sub>c</sub>: Absorbance of Glucose from the Sample; V: Total Volume of Glycogen Sample; N: Total Number of Platelets; GSK3 $\beta$ : Glycogen Synthase Kinase 3- $\beta$

### Introduction

Glycogen is abundant in the platelet cytoplasm, where it serves as a major energy source for a variety of functions, including the release reaction [1,2]. In electron micrographs, platelet glycogen appears as individual particles, randomly distributed in the cytoplasm as discrete masses of uniform size [2]. The glycogen particles seem not to exist in isolation, but are associated with enzymes involved in glycogen synthesis and degradation [3,4]. This macromolecular structure seems to be a complete functional unit in the cell, and according to some authors, should be considered an organelle, called the glycosome [1]. More recently, these structures were described as bounded by a membrane and filled with particles of glycogen [5]. Another structure present in platelets, the  $\alpha$ -granule, may occasionally contain glycogen and may be confused with glycosomes. The reason for this co-localization in these organelles is unknown [5].

The existence of large amounts of glycogen and the enzymes related to its metabolism suggests a biological role for this polymer in platelets, in addition to the possibility of storage/mobilization of the polymeric form of glucose [4]. First, platelets contain the hexokinase enzyme (ADP: D-hexose-6-phosphotransferase, EC 2.7.1.1.), which catalyzes phosphorylation of glucose to glucose-6-phosphate in the first reaction of the glycolytic pathway [6]. Both the enzymes involved in converting glucose into its polymeric form and responsible for glycogen degradation, respectively glycogen synthase (GS) and glycogen phosphorylase (GP), are found in platelets [7]. The glycogenic pathway in platelets as well as fructose-1,6-diphosphatase activity were also described. Hence, platelets are able to synthesize glycogen from lactate, and the presence of fructose-1,6-diphosphatase allows them to produce glycogen from pyruvate or citrate [8].

The presence in platelets of enzymatic machinery related to glycogen metabolism shows that this energy resource is not merely a simple heritage from the megakaryocyte. Glycogen stores in human platelets

are equivalent in amount, gram-to-gram, to those found in skeletal muscle [4], which is easily demonstrated by ultrastructure techniques, as well as by cytochemical assay [3]. However, despite the potential importance of glycogen determination in the platelets, a biochemical method to quantify this polymer in these cells is not available at the moment. The aim of our study was to establish a protocol to determine quantitatively the glycogen from human platelets.

### Material and Methods

#### Obtaining samples

Human blood was collected from 16 healthy male volunteers registered as donors in the Hemotherapy Service of the Clinical Hospital of Porto Alegre, Rio Grande do Sul, Brazil. Two samples of 4 mL were taken from each donor from the antecubital vein and placed in vacutainers (BD Franklin Lakes, USA) containing K<sub>3</sub>-EDTA.

#### Sample preparation

Immediately after the sample was obtained, the two vacutainers from each subject were gently inverted 10 times and placed in a hematology analyzer ABX Micros ES 60 (HORIBA ABX SAS, Japan) to determine the number of platelets in each blood sample. Platelets were isolated as previously described by Mangano and Schwarcz [9]. The vacutainers were then centrifuged at 300 x g for 5 min at 4°C and the platelet-rich plasma (PRP) was obtained. The supernatant (PRP) was carefully removed with a plastic tip, with care not to disturb the leukocyte layer. The volume of PRP collected from each sample was recorded and the PRP transferred to a **microcentrifuge tube**. The

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PRP was then centrifuged at  $7000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The plasma was discarded and the pellet resuspended in 0.5 mL of ice-cold 0.32 M-sucrose phosphate buffered (pH 7.4). The suspension, hereafter referred to as platelet concentrate (PC) was repeatedly passed through a plastic tip of 1 mL until the visible platelet aggregates were eliminated. An additional 0.5 mL of buffered sucrose was added to the suspension and the solution was mixed with 5 gentle inversions. The platelet suspension was again centrifuged at  $7000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The supernatant was discarded and the platelet pellet was resuspended in a volume of sucrose (0.32 M, pH 7.4 at  $4^{\circ}\text{C}$ ) equal to one-fifth of the initial volume of PRP obtained. After that, both PC suspensions obtained from each subject were blended and used for glycogen determination. The number of platelets was also determined in the platelet concentrate.

### Glycogen determination

The assay to determine the glycogen content comprised the following steps: (1) cell lyses and glycogen extraction, (2) precipitation of glycogen and its washing, (3) conversion of glycogen to free glucose, (4) neutralization, and (5) detection of free glucose.

**Step 1:** In order to lyse the platelets, 0.25 mL KOH (30%) was added and the suspension was repeatedly (15 times) passed through a plastic tip of 1 mL to homogenize the mixture. The volume was transferred to a conical tube, and another 0.25 mL KOH (30%) was added and the mixture homogenized again (15 times). For the glycogen extraction, the tube was boiled for 1 h and then 50  $\mu\text{L}$  of saturated  $\text{Na}_2\text{SO}_4$  (35%) was added.

**Step 2:** The glycogen obtained from the sample was precipitated with 1 mL anhydrous ethanol and the tube placed briefly on a vortex mixer. After that, it was centrifuged at  $750 \times g$  for 10 min, the supernatant carefully removed, and the precipitate resuspended in 0.5 mL of distilled water. The precipitation and washing steps were done once more, as described above, and the glycogen was finally resuspended in 0.3 mL of distilled water for its determination.

**Step 3:** The total glycogen obtained was converted to free glucose by acid hydrolysis. In each experiment, a standard curve (Figure 1) was established from a stock solution ( $1 \text{ mg mL}^{-1}$ ) of glycogen type III (SIGMA, USA), and different amounts of glycogen, 50 (S1), 100 (S2), 150 (S3) and 200  $\mu\text{g}$  (S4) were used as a reference. The glycogen obtained from the sample and the one that was used in the standard curve were hydrolyzed with HCl (4 N) by boiling for 1 h. In this process, 0.3 mL of HCl was added to S1, S2, S3, S4 and to the sample.

**Step 4:** After the mixture had cooled, 0.3 mL of  $\text{Na}_2\text{CO}_3$  (2 M) was slowly added to each point of the standard curve and also to the sample to neutralize the solution. This process generates large amounts of bubbles and must be done carefully.

**Step 5:** After neutralization, free glucose obtained from hydrolysis of glycogen polymer of standards (S1-S4) and sample, was determined using a glucose-oxidase kit (LABORCLIN BIOLIQUID, Brazil). For this, an aliquot of 40  $\mu\text{L}$  of sample (or of the standard curve) was incubated with the enzyme reagent for 5 min at  $37^{\circ}\text{C}$ . Absorbance was read at 505 nm.

### Glycogen calculation

The platelet number in each PC sample was used in the glycogen calculation. The equation used to calculate the amount of glycogen is given below:

$$G = \left( \frac{1}{n} \sum_{i=1}^n \frac{G_{sn}}{Abs_{sn}} \right) \times Abs_s \times V \times 10^6$$

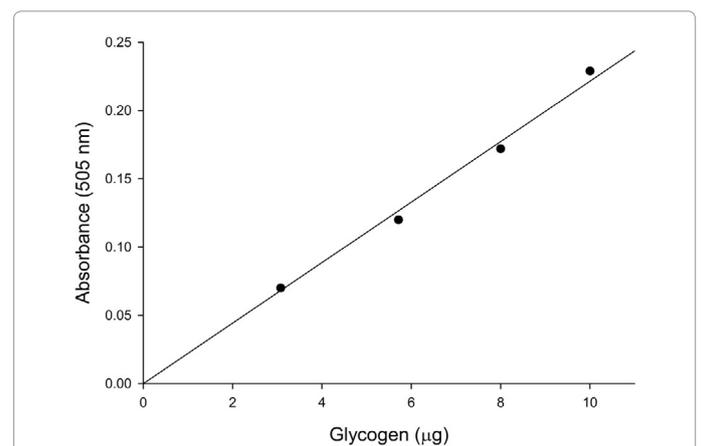
where  $G$ =glycogen ( $\mu\text{g}/10^6$  platelets);  $G_s$ =glycogen concentration

standard ( $\mu\text{g mL}^{-1}$ );  $Abs_s$ =absorbance of glucose standard;  $Abs_e$ =absorbance of glucose from the sample;  $V$ =total volume of glycogen sample;  $N$ =total number of platelets.

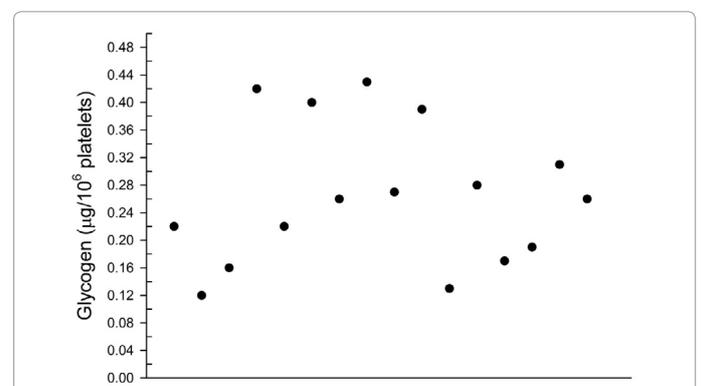
### Results

The cell count was performed in whole blood and again after obtaining the PC, using an automated hematology analyzer. The platelet number in each PC sample was used to normalize the glycogen quantity. Our data showed that platelet counting should be determined after the PC is obtained, since the cell number was reduced significantly during the process. Despite the loss of platelets, the mean platelet volume in the PC was exactly the same as measured in the whole blood,  $7.8 \pm 0.7 \mu\text{m}^3$  (mean  $\pm$  SD,  $n=16$ ). In our analysis, the platelet number achieved in the PC was  $1696 \pm 483 \times 10^3 \text{ mm}^{-3}$  (mean  $\pm$  SD,  $n=16$ ) and showed an absence of contaminant cells.

After the platelet suspension was obtained, the cells were lysed and the glycogen was extracted through incubation with KOH. The time chosen (1 h) was determined after tests of different exposure times (data not shown). The amount of glycogen quantified in human platelets was  $0.263 \pm 0.101 \mu\text{g}/10^6$  platelets (mean  $\pm$  SD,  $n=16$ ), and the data are shown in Figure 2.



**Figure 1:** Representative standard curve for glycogen. In each experiment, different amounts of glycogen (50, 100, 150 and 200  $\mu\text{g}$ ) were hydrolyzed. The values of glucose at each point were used to calculate the amount of glycogen in the sample ( $R^2=0.99$ ).



**Figure 2:** Scatter plot representing the values of glycogen obtained in the PC samples. The figure depicts the values of glycogen ( $\mu\text{g}$ ) from 16 healthy donors, normalized by the number of cells ( $10^6$ platelets).

## Discussion

The large amount of glycogen and enzymatic machinery related to glycogen metabolism found in platelets suggest that this molecule plays a significant role. Nevertheless, few studies have examined platelet glycogen. The biological function of platelet glycogen seems not to be completely understood, especially considering that these cells may respond differently under different oxygen tensions [10,11]. Another important aspect is a potential relationship between platelet glycogen stores and physiopathological conditions [12-14]. The scarcity of data regarding glycogen function in platelets could be partially attributed to the lack of a protocol to quantify this molecule in these cells. The protocol described here allowed us to quantify the glycogen from human platelets, proving to be a simple and feasible method to measure these reserves from a blood sample. Even though it uses only small quantities of tissue, the method was rapid and reliable. Notably, the glycogen, determined from glucose, could also be normalized by other parameters besides counting the number of platelets; however, if used, this determination should be done in the PC and not in whole blood.

Recently, we hypothesized that the quantification of glycogen in platelets might be used as a peripheral biomarker of glycogen synthase kinase 3- $\beta$  (GSK3 $\beta$ ) activity [15], since this enzyme may directly regulate GS (to its inactive form) through phosphorylation [16]. In addition to its pivotal role in the regulation of glycogen synthesis through GS inactivation [17-19], GSK3 $\beta$  regulates several intracellular signaling pathways, including the cell cycle, gene expression and apoptosis [20,21]. This enzyme also has essential roles in neurons, where it regulates the cytoskeletal status [21,22], and abnormal regulation of its activity has been implicated in the pathophysiology of Alzheimer's disease [19], diabetes [14] and depression [23]. Although there is no evidence that glycogen levels is a biomarker of GSK3 activity, the determination of platelet glycogen might be useful to test this hypothesis, and this polymer could be a useful parameter to evaluate biochemical changes in other pathologies. With the protocol described here, we expect to contribute to future studies of platelet metabolism, especially regarding glycogen function in these cells.

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