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IN VITRO EFFECT OF TRIGONELLINE ON THE ERYTHROCYTE AGGREGATION IN A MODEL OF DIABETES

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INTRODUCTION

In the last few decades, a large number of medicinal plants popularly used for the treatment of diabetes have been studied around the world (Chang et al., 2013). The chemical characterization of plant components and the biological, pharmacological or toxicological activity of different plant extracts have been well studied; however, more research is necessary on the relationship between phytochemical components and their specific therapeutic action. Trigonelline is an alkaloid present in the leaves of *Bauhinia sp.*, popularly known as "cow-hoof", and also in coffee and fenugreek seeds, which were used as an antidiabetic treatment by ancient civilizations. It has been found to reduce hyperglycemia, both in animals and humans, to protect pancreatic ß-cells, and to increase the rate of insulin sensitivity (Toloza-Zambrano et al., 2015).

Blood samples from diabetic patients show an alteration in the parameters of erythrocyte aggregation as well as in the mechanical properties of the red blood cell (RBC)(Riquelme et al., 2003). In addition, numerous studies in type 2 diabetes have detected important hemorheological abnormalities such as an increasing of RBC intracellular viscosity due to high levels of glycated hemoglobin. This important alteration leads to a reduction in erythrocyte deformability and a decrease in the sialic acid content of glycophorins in erythrocyte membranes, a determinant of the negative charge at cell surface, which is associated with an increase in RBC aggregation (Carrera et al., 2008). The alteration in erythrocyte aggregation is principally observed in the formation of abnormal aggregates in the form of clusters differing from the rouleaux observed in healthy donor samples. Also, alterations of erythrocyte viscoelasticity increases blood and plasma viscosity, and a higher erythrocyte aggregation degree caused generally a blood flow alterations in diabetic patients resulting in complications as diabetic foot and retinopathy (Delannoy et al., 2015).

The objective of the present work was to evaluate the hemorheological action of trigonelline on RBC in order to elucidate how extracts of these plants and/or their components exert their antidiabetic activity. This information is necessary for the design and development of new drugs to treatment of diabetes and it is essential for the formulation of possible pharmaceutical forms.

MATERIALS AND METHODS

Red blood cells (RBC): Fresh blood sample was collected from healthy donor by venepuncture in sterile vials containing EDTA as anticoagulant, with prior informed consent. Then they were washed with Phosphate Buffered Saline solution. All procedures were performed according to the "New Guidelines for Hemorheological Laboratory Techniques" (Baskurt et al., 2009).

In vitro diabetes model: Equal volumes of washed RBCs and glucose solution of the concentration 0.5 and 1 g/dL (G0.5 y G1) were incubated at 37° C for 3 hours with controlled gentle agitation obtaining gRBCs.

Treatment with Trigonelline: After washing the samples, RBCs were incubated for one hour with solutions of Trigonelline (Sigma Aldrich) in phosphate buffer solution (PBS) at 0.25 and 1.0 mg/mL (T0.25 y T1).

Digital image analysis: The digital images were obtained using an inverted optical microscope (Union Optical) coupled to a digital camera (Mikoba 300 CMOS 3.0). To do this, a drop of a RBCs suspension in autologous plasma at 0.3% was placed on a slide and after 5 minutes, 5 images of each sample were recorded as described in the literature (D'Arrigo et al., 1999).

Optical chip erythrocyte aggregometer: this device was developed in the Group of Biomedical Physics of the IFIR (CONICET-UNR). The instrument is based laser transmission technique and allow a rapid and efficient evaluation of erythrocyte aggregation phenomenon using only 15µL of blood in the chip (Toderi et al., 2017).

Digital image analysis: Shape parameter of the aggregates (ASP) and the isolated cell coefficient (CCA) were calculated from digital images using the following equation:

$$ASP = \frac{4\pi A}{p^2} \qquad C_{CA} = \frac{CA_{inicial} - CA_{final}}{CA_{inicial}}$$

RESULTS AND DISCUSSION

Samples were observed with Inverted Optical Microscope, obtaining images as illustrated in Figure 1. The images show how the globular aggregates increase with the addition of glucose solutions and decrease with the addition of T0.25 and T1, showing a great number of isolated cells and the development of rouleaux.

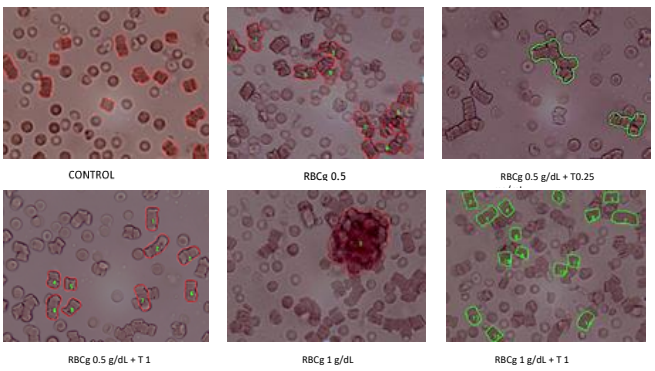

Figure 1. Images of RBCs incubated with glucose (RBCg) at different concentrations and the addition of T0.25 and T1.

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Shape parameter of the aggregates ASP and the isolated cell coefficient CCA were calculated with Image J Program.

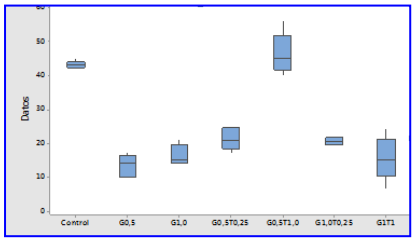

Figure 2. Percentage of isolated cells.

Figure 2 shows in a chart of boxes how in the sample of G0.5 the CCA increases with the addition of T0.25 and it is greater with T1, reaching the percentage of isolated cells in control ($p<0.01$). Table 1 shows how ASP increased and CCA decreased when adding glucose solutions, due to the formation of globular aggregates, tending to the Control values when adding T.

Table 1. Shape parameter of the aggregates ASP and the isolated cell coefficient CCA.

Sample	ASP	CCA
Control	0.64 ± 0.06	0
G0.5	0.80 ± 0.07	0.75 ± 0.06
G1	0.89 ± 0.09	0.67 ± 0.09
G0.5+T0.25	0.75 ± 0.06	0.53 + 0.08
G0.5+T1	0.61 ± 0.06	0.16 ± 0.01
G1+T0.25	0.78 ± 0.07	0.53 ± 0.03
G1+T1	0.75 ± 0.07	0.56 ± 0.12

The time to attain of half aggregation (t1/2) and aggregation amplitude (A1/2) for each sample were determined from the Aggregation kinetic curves obtained with the optical chip erythrocyte aggregometer in 400 seconds (Table 2).

Table 2. Parameters obtained from the aggregation kinetic curves.

Sample	t1	Amp 1/2	t 1/2	AI	M-Index	error
c0	80,82	71,44	111,0	0,65	26009	5%
c1	134,49	77,60	95,5	0,69	27485	5,38%
c2	190,69	69,28	123,0	0,63	25060	6,50%
G1	27,33	72,15	116,5	0,64	25644	6,34%
G1T025	161,72	68,53	128,0	0,62	24991	6,81%
G1T1	22,07	67,50	132,0	0,61	24552	6,90%
G05	154,14	75,96	102,0	0,67	26807	5,78%
G05T1	16,40	63,42	146,5	0,59	23587	7,30%
G05T025	29,02	64,42	143,0	0,59	23639	7,31%

Table 2 shows how the parameters A1/2 and t1/2 are modified by adding T to the glycated red blood cells. Aggregation kinetic curves showed a decrease of t1/2 and an increase of A1/2 in all samples treated with glucose solutions, which were reversed when T was added.

CONCLUSION

The results obtained with the *in vitro* non-enzymatic glycation treatment indicate that the aggregation parameters are modified, as evidenced in diabetes mellitus and other vascular diseases. These changes may lead to the glycation of the membrane proteins with the consequent alteration of the surface charge of the erythrocyte membrane, which could revert by the action of trigonelline. Besides that, the results obtained provide information necessary for the study of the hemocompatibility and the action of the T on the erythrocyte membrane in order to evaluate the possible alterations of the structural proteins that influence the erythrocyte morphology. These results will be useful in later studies on the possibility of the use of T as adjuvant in the treatment of Diabetes Mellitus.

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