DEVELOPMENT OF THE AGGREGOMETER FOR NEAR-INFRARED SENSORS THAT STUDY OF THE PLATELET AGGREGATION

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Abstract. *The cardiovascular disease are the causes one the major of the dead of the Brasil and this has been incentived the development of artificial devices for implanting in the cardiovascular system that deepen the knowledge process of thrombus formation, that is, thrombogenesis. This process consists of the aggregates formation of blood cells, of plasma and of fibrin. Such aggregates (thrombus) can be originated by alterations of the flow conditions and the contact with the walls of the device, that frequently can leading to the patient death. In this present work, developed three version an optic device based of the turbidimetric method that uses infrared light sensor capable to analyze the platelet aggregation, that is, to the measure that platelets agregate ones to the others, the turbidity of the plasma is modified with increase the area of pass of infrared light produced by the Light Emitting Diode (LED) that can be perceived by one phototransitor. Thus, the three version of the device is able to perform the analysis of the aggregation for the variation of the optic density in the plasma sample during the aggregation process. Although similar devices exist in the market, the devices went developed in the Bioengeneering Laboratories of the Departament of Mechanical Engineering of the Federal University of Minas Gerais (UFMG) have as main advantage to allow the evaluation of the process of aggregation in different flow conditions with more an accessible cost. The preliminary results of aggregation obtained with the three version of the device developed permited quantify to proccess aggregation for the uses of infrared sensors and went compatible to results similar device existent in the market.*

Keywords: *platelet aggregation, turbidimetric method, infrared light, hemodinamic, thrombogenesis*

1. Introduction

Coronary stenosis is a decrease of the lateral section of arteries surface which propel heart and it is provoked by the formation of fat plaque below the endothelium. That stenosis decreases the bloodstream toward the cardiac muscle and provokes severe consequences. These complications are due to the interaction between blood and the lipid plate (known as atheroma plate) formed in the vessel. When this plate bursts, it reveals sub-endothelial structures (such as collagen) which activate blood clot. That process forms embolus which causes a blockage in the post lesion and, as a result, provokes a heart attack. In this case the platelets are activated in two different moments: 1) passing through stenosis and imposing an unusual flow pattern, 2) when the lipid plaque bursts, reveals the collagen and it develops the process of adhesion/aggregation of platelets and the formation of fibrin causes thrombus.

Initial studies on the thrombus formation process or thrombogenesis were carried out in 1856 by Virchow and, since then, it has been known that the phenomenon occurs due to the flow, the blood biochemistry and the surfaces in which the blood may be in contact (PETSCHEK, 1968). Clot activation and thrombus formation occur when the events known as Virchow triad are initiated: 1) the activation of platelets; 2) the activation of clot factors; and 3) the activation of the fibrinolytic system.

By using in vitro studies with blood, the formation of vortex, originated from a stenosis, is characterized by increasing the shock between the platelets; due to long periods and radial collision toward the wall vessel (KARINO et al., 1987). This process favours the thrombus development, without adding aggregation agents, or rather, vortex may cause hemolysis (red globules burst) by liberating the inductor agent of aggregation, Adenosine Diphosphate (ADP), and activating the platelets. Vortex may conduct to thrombus and thrombosis formation (STEIN and SABBAH, 1974).

One way of studying the aggregation process of in vitro platelets is carried out by the stirring of a suspension of Platelet Rich Plasma (PRP). Once the platelet aggregation is started (in consequence of its activation) the suspension speed decreases provoking a change of the infrared light quantity absorbed by the plasma sample. Due to this reason the stirring method of a PRP suspension is called Method Turbidimetric.

The study on the potential of the *in vitro* platelet aggregation, or rather, the capability of platelet aggregation when submitted to either physical or chemical stimulation, as well as the diagnosis method in the area of tissue engineering,

have employed the infrared spectroscopy as sensors (HAZEN et al., 1998); holding the great promise for noninvasive, nondestructive and therefore less aggressive method (HAZEN *et al*,1998, FINE and SHVARTSMAN, 2003).

Infrared sensors work according to the absorption frequency of each one of the different blood cells. It makes them able to detect any blood structural alteration, specially the cellular adhesion and aggregation which alter as the blood is submitted to different conditions of flow and/or exposed to different kinds of surfaces (BERGER et al., 1997).

Near-infrared spectroscopy is one the three invisible light of the infrared electromagnetic spectrum which has been used in both tissue and cell stimulation. Near-infrared spectroscopy – NIR – is based on the absorption of electromagnetic radiation with wavelengths raging from 750 - 2500nm, or rather, it is a radiation close to the visible light spectrum (HAZEN *et al.*, 1998). The radiation which interacts with a sample may be absorbed, transmitted or reflected. Reflectance is eliminated so the relation between non-absorbed and transmitted light intensity –from the sample– can be measured as the transmittance and the absorbance as the quantity of absorbed light.

LEDS (Light Emitting Diode) and the photosensors sensible to emitted radiation appeared as some of the near-infrared light device, able to analyse blood. It has been verified the development of high intensity LEDS, which have low spectral rate, with specific wavelength in the near-infrared area and low cost.

Different optical and electrical devices, able to monitor change in blood sample, were developed to the study on platelet aggregation. One of them was the equipment developed by Feinman *et al* (1977), which allowed to monitor both answers given by the stimulated platelets: ATP aggregation and secretion. This device works according to the already shown turbidimetric method, however, its flow induction speed is steady. This system of aggregation measurement uses a 55C LED (General Electric) with a 940 nm emission spectrum and a FTP-100 phototransistor (Fairchild). Another technique used to *in vitro* analyses consists of detecting the potential difference obtained by the insertion of electrodes into the sample (CARDINAL and FLOWER, 1982; FREILICH, 1986). There is another technique of cellular monitoring, of platelet aggregation and of variations in other parameters of platelet aggregation, which uses a device with a tungsten lamp to create visible light on the filter. For that reason, the method allows to analyse the electromagnetic radiation at definite wavelengths (RENAUD and RUBEL, 1978). Davis (1968) developed a measure technique of platelet aggregation and clotting in which both the turbidimetric method and the Chandler tube are used. The technique consists of adapting a photospectroflowmeter to a 620nm monochromatic filter. Fukuyama *et al* .(1989) developed an optical device to promote shear-induced platelet aggregation (SIPA). It was done based on a turbidimetric technique as employed in commercial platelet aggregation. In this process the optical density of the PRP sample was detected by a (wavelengths 633 nm) laser light source and a sensor, both guided by an optical fiber.

The degree of platelet aggregation has been used as a sign of some diseases of cardiovascular system or some blood biochemical disorders in clinical studies. The developed device may be used to: 1) investigate some hemorrhagic diseases, i. g., Glanzmann's thrombasthenia, von Willebrand's disease and Bernard-Soulier disease; 2) monitor the results of using platelet disaggregates on patients who present platelet hiper-activation; and 3) distinguish those individuals who present a platelet hiper-activation from the others, pointing out the necessity of prophylactic initiatives in order to avoid thrombus formation.

The purpose of this study is to developed a Optical Aggregometer which quantifies the platelet aggregation in equine blood plasma by the Turbidimetric Method, the LED/Phototransistor infrared sensors and variable speed. The experimental tests were compared to a commercial equipment which works on the same principles.

2. Methods

2.1 Construction of the device

A device was developed due to the need of studying the flow effects on platelet aggregation by the turbidimetric method as shown in Figure 2.1.

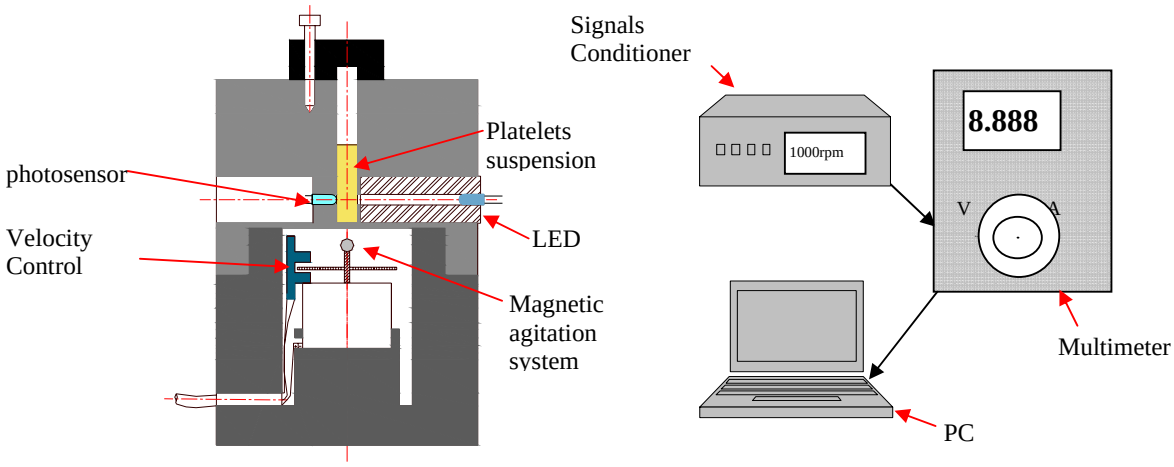

Figure 2.1: Squematic draw of the aggregometer and data acquisition system used that to study of the Platelet Aggregation process

Speed control, data acquisition system (PC, Multimeter and signal conditioning equipament) are turned on to activate the stirring system from 300 to 2000 rpm. Due to the necessity of simulating different flow conditions, the speed can be manipulated. Later, a sample of platelet suspension is placed in the aggregometer channel and closed to avoid surrounding light.

Turbulence data is read as the optical density difference of the PRP sample is transformed into transmittance sign by LED/phototransistor optical pair. It occurs as the sample is stirred to quantify this sign as a Platelet Aggregation percent (% Ag) in terms of time. In this way, platelet aggregation is represented.

Transmittance sign is sent to a conditioned plate to transform this sign into tension sign, which is sent to digital multimeter. A mobile computer (PC) receives this last sign through a serial port and records that data obtained by software Masview version 1.1.

The conditioning and amplification system of the sign from LED/photosensor optical pair is done by means of a micro-controller PIC16F876. There is another micro-controller to monitor the 12 Volts and 3 amperes electric motor (Tenko Motors). Motor speed control is done by an encoder model PHT18 (Texas Instrumenys).

Comparative tests of platelet aggregation were carried out with a Packs-4 Aggregometer (Helena Laboratories).

2.2 Aggregation measurements

Data was collected using infrared light sensors, spectral scale 880 nm. The transduction system is compound of LED infrared diode or TIL32 emission (Texas Instruments) and of a phototransistor or receptor model TIL78 (Texas Instruments) sensible to the emitted radiation.

Magnets with the plasma sample are required to the performing of the magnetic stirring system. The magnets used in PRP homogenisation from Labbio aggregometer were chosen due to their magnetic intensity, what is fundamental to the development of the stirring system of Labbio aggregometer. The lower cost of Labbio in comparison to Packs-4 aggregometer was also considered.

2.3 Samples and reagents

Blood samples were collected from 13 adult equines. 20 ml plastic syringes (40 x 16) contained 2 ml of Sodium Citrate anticoagulant (volume of 3,8%) were used in order to minimize hemolysis. Among those equines, six were under experimental conditions of induced laminitis. Laminitis is a disease which attaches the animal immune system, provoking a platelet pre-activation. The other seven animals were healthy.

Blood samples were collected and prepared as stated by Standard ISO 10993-4 (SEYFERT et al., 2002); since red blood cells may be burst during procedures. After blood collecting, samples were processed to obtain platelet-rich plasma (PRP) and platelet-poor plasma (PPP).

Some tests were carried out without using ADP, in order to study spontaneous platelet aggregation when it is submitted to more turbulent flow conditions. Spontaneous aggregation concerns platelet aggregation provoked by the mechanic shock caused by platelets. The process initiates with platelet membrana bursting what releases ADP in the dense granules of platelets. ADP activates the liberation of Calcium ions from the dense tubular system to feed the cycle of platelet activation. However, ADP was used in a majority of the tests to accelerate the activation and aggregation platelet process.

3. Results and Discussion

3.1. Optical model applied for the Aggregometer

The optical model shown in the section (OLIVEIRA,2004) was developed based on the change of optical density in plasma samples, which occurs as platelets aggregate. This aggregation allows a larger light passage. From the relation between light quantity emitted by photodiode and perceived by photosensor arises a potential difference called transmittance.

In the analyses of transmittance data (section 3,2), some conditions were assumed:

1. Radiation absorbed by cells is meant to be proportional to the cell surface and transversal to the direction of radiation incidence.
2. Spread radiation is absorbed by either other cells or small tube walls.
3. Spread is meant to be proportional to the cell surface and transversal to the direction of radiation incidence.
4. Platelet surface in the suspension can be calculated based on the total number of platelet in the suspension.
5. Transmitted radiation is inversely proportional to the cell number in the sample.

According to these conditions, optical density can be represented by the number of cells in the sample, or rather, volumetric cellular density. Therefore, the optical density of the sample can be represented cells mean surface by volume unit; since the transversal surface to the beam is the source of absorption or spread of radiation.

An initial moment of analyses is assumed as a time reference in order to calculate platelet aggregation percentage of any suspension, at any time. A calculation of the number of platelet is carried out at the initial moment (No). Later, the same calculation is carried out to greater moments than the initial one (Np). Theoretically, the lowest platelet number of plasma suspension is the lowest platelet number of a PPP plasma suspension (Nppp). Therefore the greatest variation of platelet number in any plasma suspension would be (No-Nppp). Any variation between the initial moment and any other time would correspond to a percent of this number. It is considered that total surface of platelet (Ap) can be calculated as the surface of one platelet times the platelet number in the suspension.

The relation platelet number in the plasma sample with the Aggregometer data can be obtened for eq. 3.1, where the platelet number is function tension sign read in the aggregometer of the that form:

$$N_p = N_{PPP} \cdot \left[1 + \frac{1}{\alpha} \left(1 - \frac{V_{PRP}}{V_{PPP}} \right) \right] \quad 3.1$$

Of the eq. 3.1, verified that number of platelet is carried out at the initial moment can be represent for eq. 3.2:

$$N_0 = N_{PPP} \cdot \left[1 + \frac{1}{\alpha} \left(1 - \frac{V_0}{V_{PPP}} \right) \right] \quad 3.2$$

Therefore, the percent of aggregation is calculated by the eq. 3.3:

$$\%Ag = \frac{(V_{PRP} - V_0)}{(V_{PPP} - V_0)} \cdot 100 \quad 3.3$$

3.2 Results of Platelet Aggregation

3.2.1 Results of Platelet Aggregation to Labbio Aggregometer

The first test carried out with Labbio Aggregometer consisted of testing the sensor ability in detect optical density changes in PRP samples by the formation of platelet aggregation when submitted to different conditions of flow. Or rather, mechanical aggregation tests were carried out without using ADP (Fig. 3.1). In these tests, PRP samples were submitted to different conditions of flow by speed variation. It was noticed that, to the three shown curves, there is a phase of platelet activation which occurs and is characterized by the change of platelet forms. In the aggregation phase, characterized by the increase of transmittance, the intensity at witch platelets aggregate do not vary. It occurs because the shear-stress rate imposed is sufficient to burst the platelet membrana and release the Calcium ions.

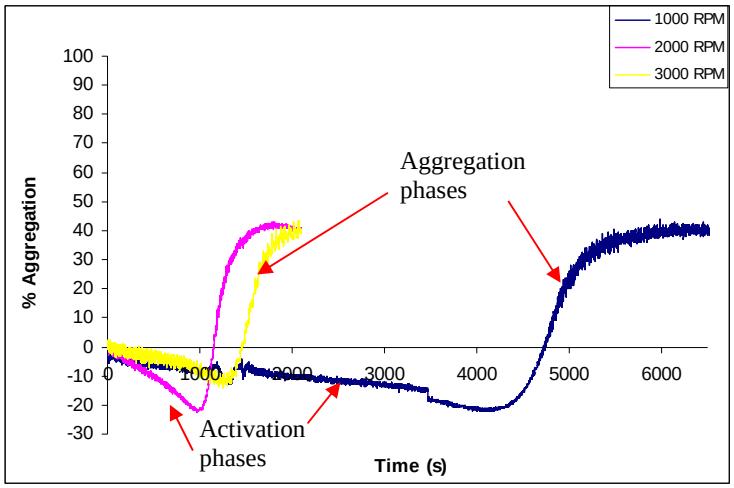

Figure 3.1: Mechanical aggregation curve obtened with Labbio Aggregometer in speed variation

Figure 3.2 shows curves of mechanically induced platelet aggregation when submitted to 3.000 rpm . Aggregation and activation phase can be observed on both curves. However a disaggregation process can be verified at the end of each aggregation curve. It can occurs for two reasons: 1) the lack of ADP makes the platelets return to their discoid form. 2) shear rate may provoke a microaggregate formation.

Tests to obtain curves of platelet aggregation induced by ADP and steady-state velocity of 1000 rpm are shown in Figure 3.3. The three curves are in the normal rate of aggregation (30 – 70%). Activation phases caused by a quick decrease in transmittance is noticed after ADP injection, about 60 seconds after the begining of the test. How platelet aggregation is accelerated with ADP using is verified now.

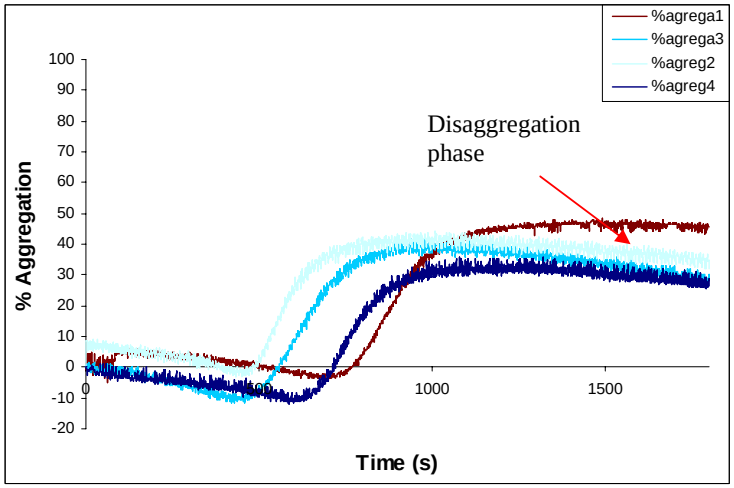

Figure 3.2: Mechanically Induced Platelet Aggregation with the Labbio Aggregometer using the same PRP sample in steady-state velocity of 3000 rpm

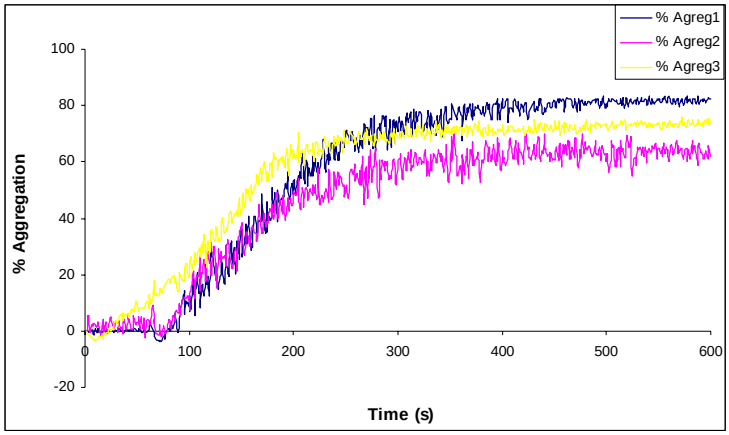

Figure 3.3: Induced Platelet Aggregation curve for ADP obtened with the Labbio Aggregometer using the same PRP sample and steady speed of 1000 rpm

Figures 3.4 and 3.5 show high levels of fluctuation during aggregation measurements. Figure 3.4 shows high percent of aggregation due to the fact that the animal had Laminitis, or rather, since the platelets were pre-induced, ADP adding might have amplified the aggregation process. This fluctuation might have been accentuated by the high number of platelets in PRP samples.

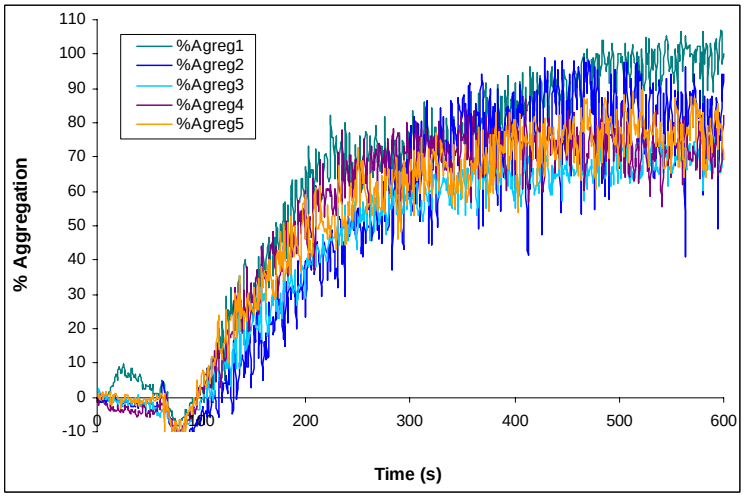

Figure 3.4: Induced agregation curve for ADP obtened with Labbio Aggregometer the steady speed of 1000 rpm using the same PRP sample of the animal had Laminits

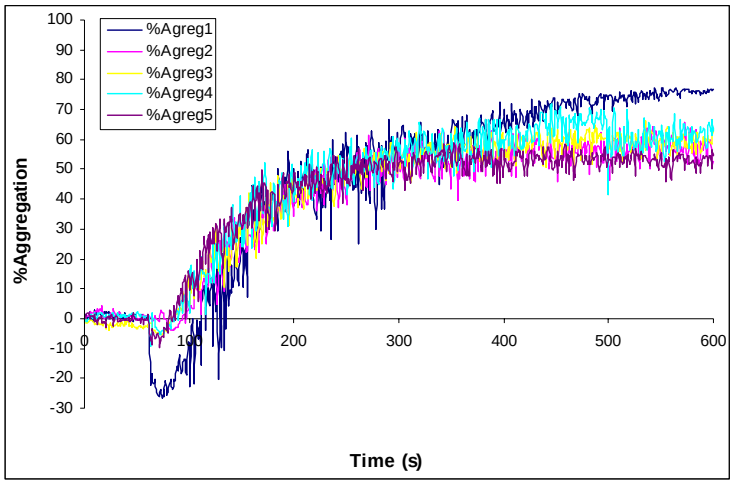

Figure 3.5: Induced aggregation curve for ADP with Labbio Aggregometer using the same PRP sample and steady speed of 1000 rpm

Figure 3.6 shows the comparison between the number of platelet and greatest aggregation of tests which were carried out with Labbio and Packs-4 aggregometers. It was noted that the preliminary results revealed by the developed aggregometer show a good correlation with Packs-4. Although the developed system reveals a higher rate of aggregation. Preliminary results of the prototype of the developed aggregometer reveal the capability of infrared light sensors – model LED/Phototransistor – to detect Platelet Aggregation. ADP induced aggregation is more accelerated and amplified than mechanical aggregation. However, ADP stored in platelets is sufficient to provoke their aggregation, what depends on the flow conditions in which platelets are submitted as time goes by.

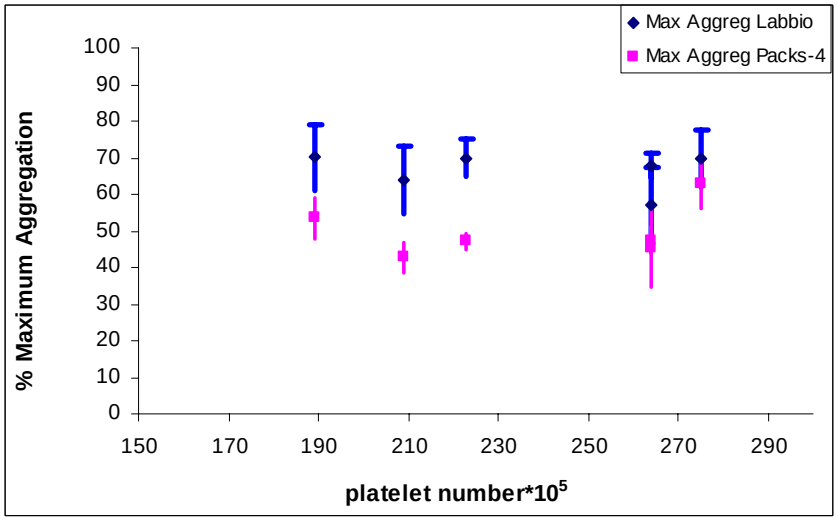

Figure 3.6: Comparison between the number of platelet and greatest aggregation of tests with Labbio and Packs-4 aggregometers

4. Conclusion

An optical aggregometer which uses the Turbidimetric Method was developed by LED/ Phototransistor infrared sensors to analyse the process of *in vitro* platelet aggregation. The experimental methodology allowed to obtain needed values to the determination of platelet aggregation. This process permitted checking the performance of Labbio aggregometer by comparing Labbio to commercial aggregometer Packs-4, which is on the market.

The measurement system by light sensor with radiation in the near-infrared spectrum model LED/Phototransistor, wavelength 880 nm, was able to detect the changes in optical density from plasma samples during the aggregation process.

The system of magnetic stirring of platelet suspension was efficient, since speed could be varied. Therefore, two facts were verified: 1) the influence of speed on the system of plasma stirring; 2) the preliminary results agree with the results obtained with the device on the market. An acceptable deviation between the devices was not statistically established due to the insufficient number of tests.

In relation to preliminary experimental tests, it was observed that the rate of platelet aggregation suffers great influence from speed and, as a consequence, the degree of turbulence imposed by the flow tends to increase aggregation. However, from a given speed, the rate of aggregation seems do not alter any longer and the platelet aggregates begin to be sheared by a turbulent flow.

The time and the magnitude of speed in which platelet aggregates are submitted to a turbulent flow determine the size of aggregates. These aggregates tend to be great to flows in which the stirring system of platelet suspension is the least non-invasive one.

Therefore, this paper is a very important step to the study on *in vitro* aggregation in terms of platelet number and flow conditions in which platelets are submitted.

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