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Microplate Reader Analysis of Triatomine Saliva Effect on Erythrocyte Aggregation

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Our hypothesis is that the action of aggregating and disaggregating substances in the blood can be detected and quantified by the Microplate Reader. To ascertain the validity of this hypothesis, we selected two types of blood: one that naturally presents erythrocyte aggregation (pig blood) and the other that does not present aggregation (bovine blood). One important reason for the choice of pig blood is that its erythrocyte aggregation resembles that of human blood. *T. infestans* saliva was added to the pig blood as a disaggregating substance, while bovine fibrinogen was added to the bovine blood as a substance that promotes erythrocyte aggregation. We investigated the dynamic viscosity (η) of these mammals' blood, of *T. infestans* saliva and of the absorption (A) by Microplate Reader, carrying out UV-Vis spectrophotometric assays of pig plasma with different concentrations of triatominae saliva and of bovine blood with different concentrations of fibrinogen. Our findings indicate that spectroscopic techniques such as the Microplate Reader complement and expand the study of blood rheology, erythrocyte sedimentation and aggregation.

Keywords: *erythrocyte aggregation, hemorheology, hematophagous saliva, Triatoma infestans, fibrinogen*

1. Introduction

Blood consists of a concentrated suspension of particles with a non-Newtonian behavior, i.e., its dynamic viscosity η ($\eta = \frac{\tau}{\dot{\gamma}}$, where τ is the shear tension) depends on the shear rate ($\dot{\gamma} = \frac{d\gamma}{dt}$) imposed by the endothelial walls on the fluid, and it is thixotropic, because viscosity *decreases* as $\dot{\gamma}$ *increases*. Unlike birds and reptiles, the erythrocytes of mammals are devoid of nuclei. Cellular structures like the nucleus, mitochondrion, etc. increase the rigidity modulus G of a micrometric particle. Thus, mammalian blood is a typical example of a deformable particle suspension. This deformability affects the viscosity, but the thixotropy of blood originates from the ability human erythrocytes have to aggregate. At low flow velocities, particularly in the arteriole and postcapillary venules, the red blood cells of various mammals (e.g., humans, horses, pigs, rodents, etc.) tend to form aggregates called *rouleaux*. These *rouleaux* look like a stack of coins, growing in size and spreading out as $\dot{\gamma}$ decreases.

2. Literature Review

Curiously, some mammals do not present this aggregation in physiological conditions, as in the case of bovines and birds[3,15]. Some authors classify mammals whose erythrocytes aggregate as *athletic animals*[7]. There is a consensus that erythrocyte aggregation is related with the content and concentration of macromolecules present in blood plasma, especially fibrinogen[5,6,9] but *how* RBC-RBC adhesion occurs and the RBC-fibrinogen interaction itself are still topics of discussion[8]. The *rouleaux* of RBC are reversible because, as the blood circulation velocity increases, they come apart without apparently harming the integrity of the RBC[14]. Erythrocytes have a limited lifetime (~ 120 days) in the bloodstream[4], and there seems to be a tendency for greater aggregation in older RBC[2]. The viscosity and aggregation of erythrocyte are features to be considered in the evaluation and evolution of a considerable number of pathologies that affect human beings, such as diabetes, hypertension, sickle cell anemia, etc.[11].

In this and previous works[1], we proposed a *new area of study* of erythrocyte aggregation, which consists of investigating: a- If the food of hematophagous insects is affected by erythrocyte aggregation, and b- The action of these insects' saliva on erythrocyte aggregation. Some of these insects are vectors of epidemics such as Dengue, Chagas Disease, Malaria, etc. A mechanical adaptation of the buccal system and the salivary composition helps these insects find and ingest blood. The saliva they release throughout the feeding process contains anticoagulants, anti-aggregating platelets and vasodilators that help them obtain a greater volume of blood[12].

Since many species of mammals present erythrocyte aggregation, we assume that triatominae species must possess substances that reduce the aggregation and viscosity of the blood of parasitized animals, facilitating the flow of ingested blood through their micrometric feeding canal (diameter of approximately 10 μm at the apex).

The objective of this work was to quantify the alterations the saliva of hematophagous insects (*T. infestans*) causes in the rheology and erythrocyte aggregation of pig blood samples using the Microplate Reader. Pig blood was chosen because its erythrocyte aggregation is similar to that of human blood, while bovine blood was chosen as a non-aggregating blood model, and the Microplate Reader because it is an optical device widely used in biological research. A major advantage of the Microplate Reader is that it requires small quantities of samples (microliters) and allows a large number of assays to be done on one plate.

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3. Experimental Procedure

3.1. Pig and bovine blood samples

The blood used in this study came from two slaughterhouses located in metropolitan Belo Horizonte, state of Minas Gerais, Brazil. The blood samples, which were collected from the jugular vein of pigs and bovines by sanitary inspectors of the Ministry of Agriculture immediately prior to their slaughter, were placed in 50 mL Falcon tubes with EDTA anticoagulant and immediately stored under refrigeration (approximately 10 °C). No approval was required from an Ethics Committee because the blood was taken from healthy animals that were being slaughtered for sale.

3.2. Triatoma infestans saliva samples

Fifth stage and adult *Triatoma infestans* were supplied by the Laboratory of Hematophagous Insect Physiology of the Federal University of Minas Gerais Institute of Biological Sciences, and by the René Rachou Research Center of the Oswaldo Cruz Foundation, both located in the city of Belo Horizonte. The saliva was collected from the tip of the insect's proboscis using a Pasteur pipette. The droplets thus obtained were placed in polymer tubes (1.5 mL), which were stored in ice and frozen (~ 18 °C) immediately thereafter.

3.3. Viscosity measurements (η – mPa.s)

The viscosity experiments were conducted in two devices: i- a BROOKFIELD model DVIII concentric cylinder-type viscometer, and ii- a BROOKFIELD model DVII+ cone-plate viscometer. The temperature of the samples was controlled during the experiments with a BROOKFIELD model TC 500 thermostatic bath (20 or 39 °C). The hematocrit was adjusted to 40% in the viscosity assays. Prior to the assays, the equipment was calibrated with CANNON and BROOKFIELD oil viscosity profiles.

3.4. Blood microstructures

The microstructure of the blood was analyzed under a LEICA DM LS microscope coupled to a MOTICAM 480 digital camera. Using MOTIC IMAGES ADVANCED 3.0 software, we were able to take measurements of the samples on a micrometric scale. In the analyses of blood and saliva microstructures, the proportions of saliva diluted in the blood were 5, 15, 25 and 50%.

3.5. Microplate reader experiments

A Benchmark Microplate Reader (Bio-Rad Laboratories) was used in the assays, which were carried out in multi-well plates (96 wells) of 100 µL each. The well plates were concave with a flat bottom. After fixing the plate on the holder, it was swirled at a shear rate not specified by the manufacturer. The overall reading time, which was programmed by specific software, took a maximum of 2400 seconds. The proportions of saliva diluted in the blood were 5, 15, 25 and 50% v/v.

3.6. UV-Vis Spectrophotometry assays

The pig plasma was analyzed using a Hitachi model U-3000 U-Vis spectrophotometer equipped with approximately 1 cm long optical quartz glass cells.

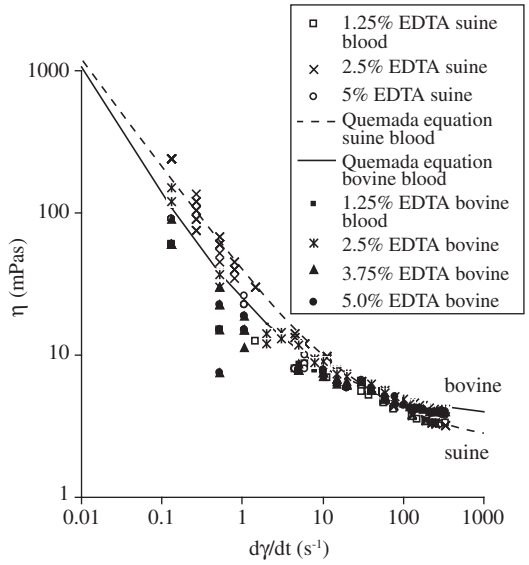
4. Results and Discussion

4.1. Bovine and pig blood viscosity

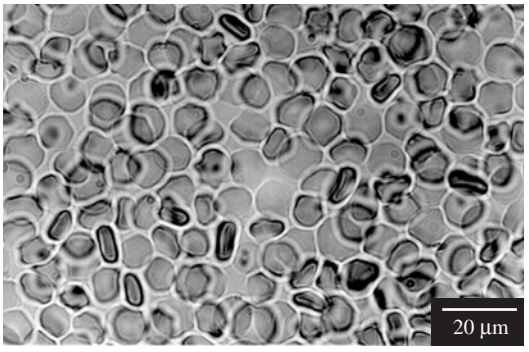
Figure 1a illustrates the assays of pig and bovine blood viscosity (η) as a function of shear rate ($\dot{\gamma} = \frac{d\gamma}{dt}$) at a temperature of 39 °C. The viscosity data were obtained by the concentric cylinders technique. Bovine and pig blood consists of suspensions of deformable particles with a non-Newtonian behavior, but having a distinct rheology, as indicated by the theoretical viscosity curves. These curves were obtained using an equation proposed by D. Quemada[10]:

$$\eta = \eta_{\infty}\left[\frac{1+\theta}{\chi+\theta}\right]^{2} \quad (1)$$

where η_{∞} is the limit value of viscosity when $\dot{\gamma} \to \infty$, θ is an adimensional constant equal to $\theta = \left(\dot{\gamma}.t_C\right)^{\frac{1}{2}}$, and $\dot{\gamma}_C = t_C^{-1}$, where t_C^{-1} can be understood as a typical disaggregating period. The exponent p ~ 1/2 and $\chi = \left(\frac{\eta_{\infty}}{\eta_0}\right)^{\frac{1}{2}}$ is called a structural index, while η_{∞} is the ultimate



(a)



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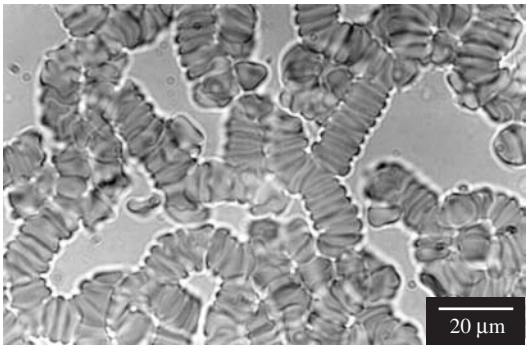


(c)

Figure 1. a) Viscosity of pig and bovine blood at 39 °C; b) bovine erythrocytes do not aggregate; and c) pig erythrocytes aggregate to form *roleaux*.

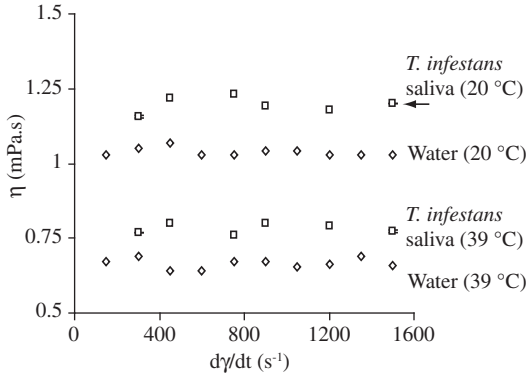
viscosity in the laminar regime and η_0 is the ultimate viscosity when $\dot{\gamma} \to 0$.

The experimental viscosity data indicate a difference in the rheological behavior of the two types of blood (bovine and pig). This difference is most evident at higher $\dot{\gamma}$ values and can be attributed to two factors: aggregability and deformability of the erythrocytes[13]. The red blood cells (RBCs) of pig, as well as of humans, are quite deformable and they aggregate. Bovine erythrocytes (Figure 1b), on the other hand, do not aggregate and, viewed under an optical microscope, appear to be less deformable than pig erythrocytes (Figure 1c). Thus, at high shear rates, pig erythrocytes will deform more towards flow than bovine red blood cells, indicating that the ultimate viscosity (η_∞) of bovine blood is higher than that of pig blood.

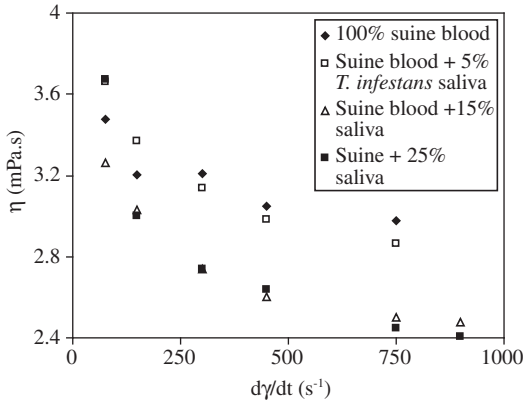
The addition of different concentrations of EDTA seemed not to significantly affect behavior of erythrocytes, since different concentrations did not alter either of the blood types.

4.2. Viscosity of T. infestans saliva and of pig blood + T. infestans saliva

In Figure 2a shows the results of viscosity η (mPa.s) *vs.* $\dot{\gamma}$ of *T. infestans* saliva samples (20 and 39 °C), and gives viscosity measures of distilled and deionized water. The viscosity η of *T. infestans* saliva and water did not vary with dγ/dt, since these are Newtonian liquids. The viscosity of the saliva declined by almost 50% as the temperature rose from 20 °C to 39 °C.



(a)



(b)

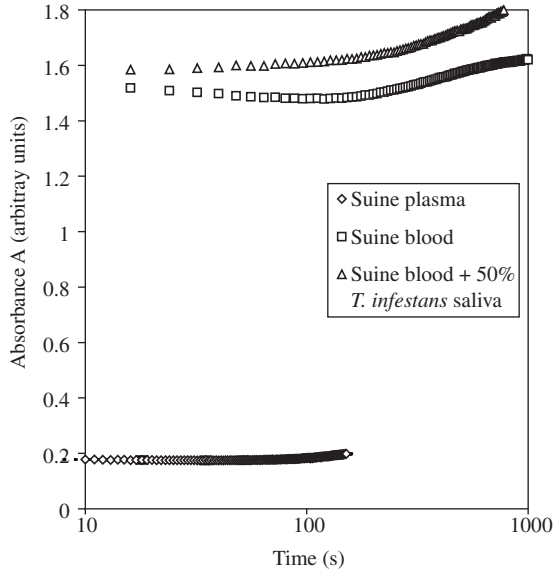
Figure 2. a) Viscosity of the *T. infestans* saliva at 20 °C and 39 °C; and b) Viscosity of pig blood with addition of *T. infestans* saliva.

The addition of 5% of *T. infestans* saliva reduced the η (in mPa.s) of pig blood (Figure 2b). The addition of 15 or 25% of triatominae saliva in the blood caused a drop in the η value at all shear rates, although this decrease was greater at higher shear rates.

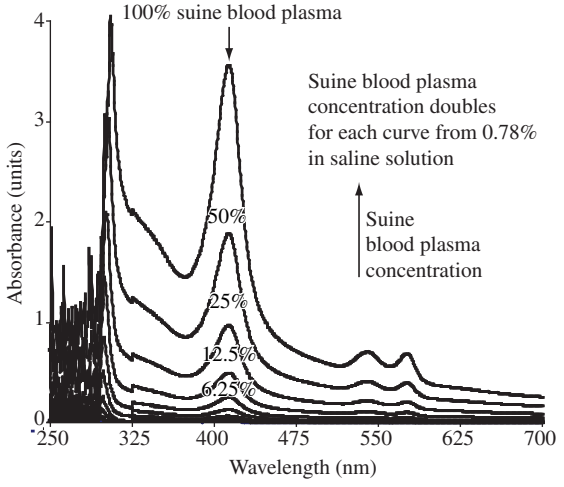
4.3. Analysis of erythrocyte aggregation using the Microplate Reader (Enzyme-Linked Imunosorbent Assay)

4.3.1. Case 1: Pure pig blood, and pig blood with *T. infestans* saliva

Figure 3a presents the absorption, **A**, at the λ = 655 nm wavelength as a function of time (in seconds) of pig blood samples (hematocrit = 10%), pig plasma and pig blood with the addition of 50% v/v of saliva. Pig plasma absorption did not affect the study of erythrocyte aggregation because the protein content of plasma does not present absorption bands at this wavelength, a fact confirmed by the UV-Vis



(a)



(b)

Figure 3. a) Microplate Reader absorption of pig blood, pig plasma and pig blood with 50% v/v of *T. infestans* saliva; and b) UV-Vis Spectrophotometer spectrum of pig plasma at different concentrations.

spectroscopic image shown in Figure 3b. The addition of *T. infestans* saliva to pig blood caused disaggregation of the *rouleaux* and altered the shape of the erythrocytes, as depicted in the micrographs in Figure 4. The addition of saliva increased the absorption because, when separated, the erythrocytes increase the total absorption area.

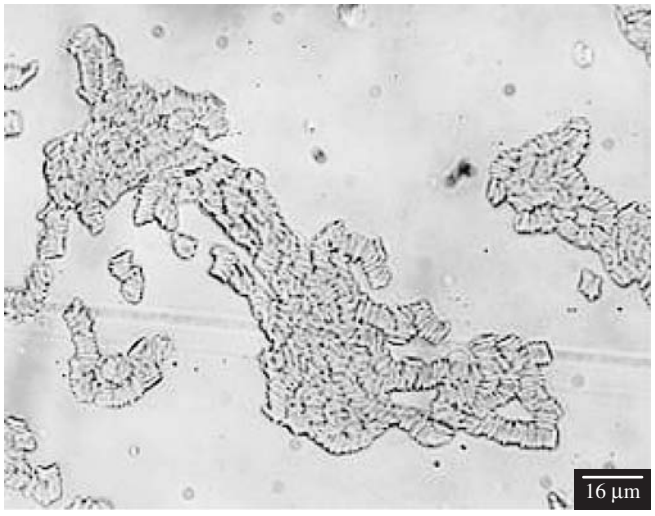
4.3.2. Case 2: Pure bovine blood, and bovine blood with bovine fibrinogen

Figure 5a shows the absorption, **A**, at the $\lambda = 655$ nm wavelength as a function of time of the bovine blood samples (hematocrit = 10%) with the addition of different concentrations of fibrinogen. Figure 5b presents the results for samples of bovine plasma, bovine plasma with the addition of 25 g/L of fibrinogen, and empty microplate wells (without samples). These results indicate that neither bovine plasma nor bovine fibrinogen affected the absorption of the samples at the studied wavelength.

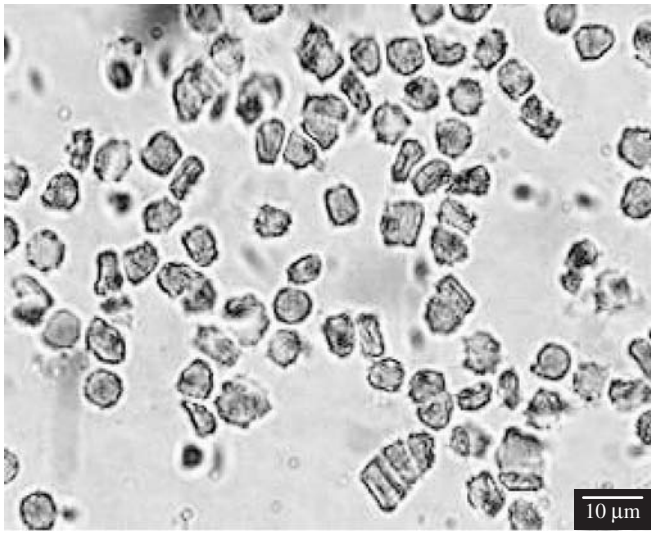
An analysis of Figure 5 indicates that the erythrocyte aggregation can be quantified by studying the tangents of the curves described by the experimental points. Figure 6 presents a sequence of images of normal bovine blood to which increasing amounts of bovine fibrinogen were added (up to 25 g/L). Note the formation of erythrocyte *rouleaux*. A more detailed analysis indicates that increasing the fibrinogen concentration affects the dimensions of the red blood cells (RBC).

5. Conclusions

The phenomenon of erythrocyte aggregation of pig and bovine blood was investigated by two distinct techniques, one mechanical (viscosity) and the other optical (Microplate Reader). This study also provided information on erythrocyte deformability and the alterations in these cells resulting from the addition of substances to the blood of these mammals.

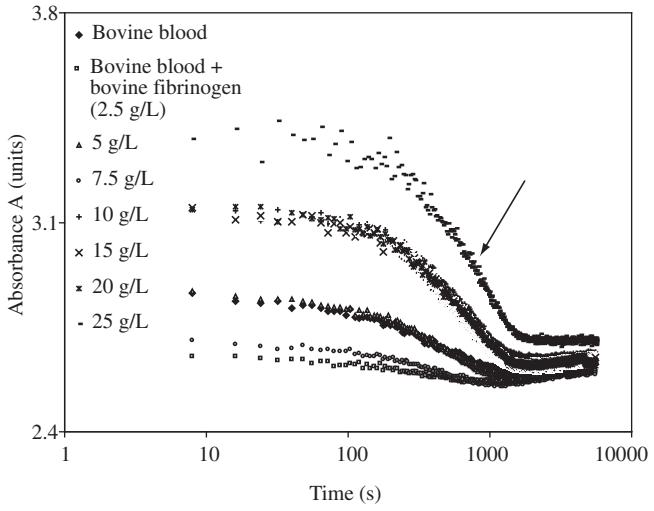


(a)

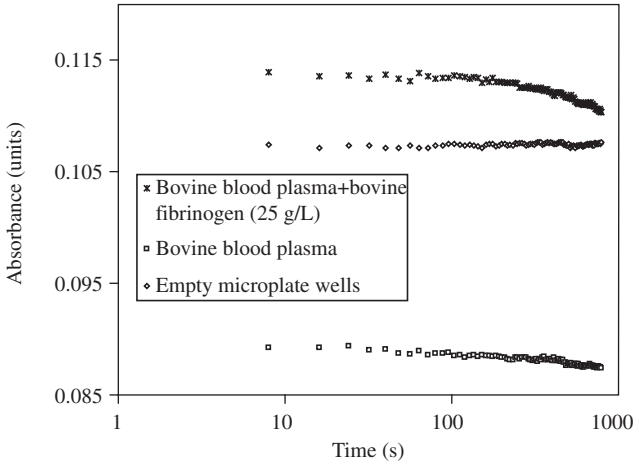


(b)

Figure 4. a) Microstructure of pig blood; and b) Microstructure of pig blood containing 50% (v/v) of *T. infestans* saliva

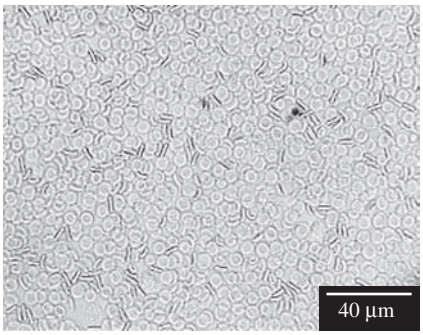


(a)

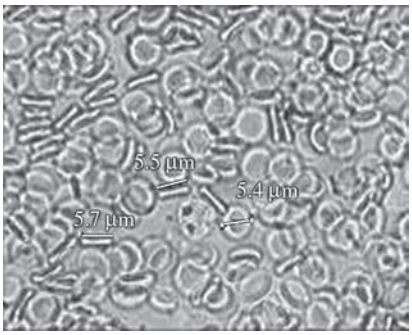


(b)

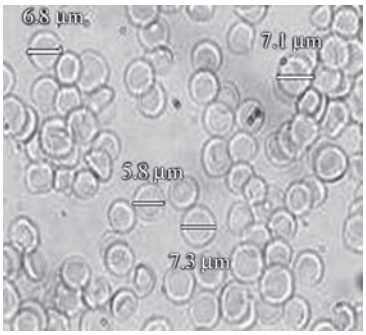
Figure 5. a) Microplate Reader: Total absorption of bovine blood, and absorption with the addition of fibrinogen; and b) fibrinogen did not affect the absorption of samples at the wavelength studied (655 nm). The absorption of plasma was lower than the absorption of empty wells on an empty microplate.



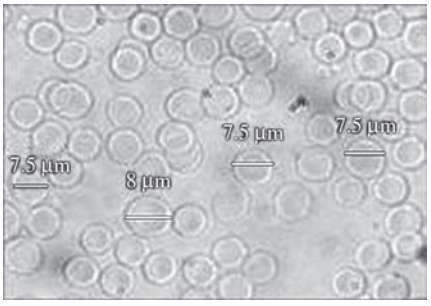
(a) Normal



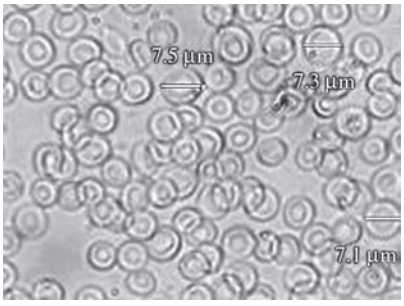
(b) Normal



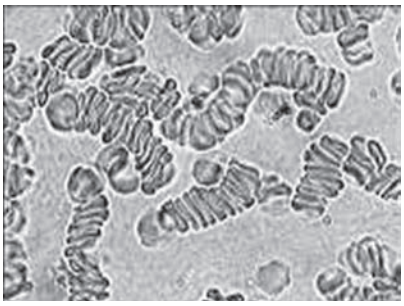
(c) 2.5 g/L



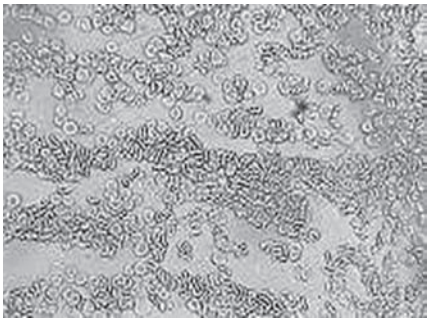
(d) 5.0 g/L



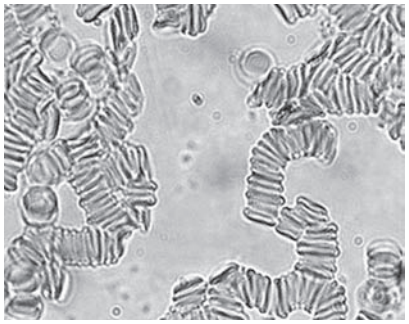
(e) 7.5 g/L



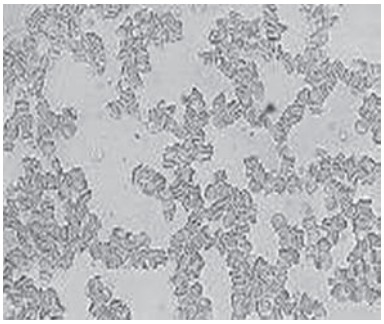
(f) 10 g/L



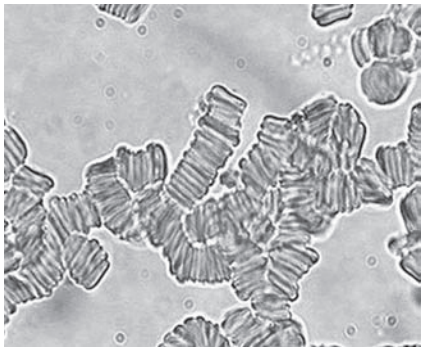
(g) 10 g/L



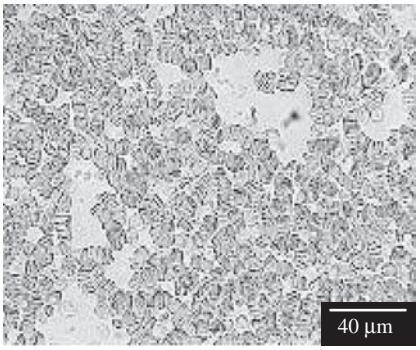
(h) 15 g/L



(i) 20 g/L



(j) 20 g/L



(k) 25 g/L

Figure 6. a,b) Microstructure of bovine blood; c) Microstructure of bovine blood containing 2.5 g/L of bovine fibrinogen; d) 5.0 g/L; e) 7.5 g/L; f,g) 10.0 g/L; h) 15 g/L; i,j) 20 g/L; and k) 25 g/L.

Pig blood is similar to human blood and presents erythrocyte aggregation and deformability. In contrast, the erythrocytes in bovine blood do not aggregate and the red blood cells (RBC) display less deformability. Our viscosity assay confirmed this qualitative evaluation, since $\eta_{\infty bovine} > \eta_{\infty suine}$ at the ultimate viscosity. *T. infestans* saliva is a Newtonian fluid whose viscosity is very similar to that of distilled water at a temperature of 39 °C (~ 0.5 mPa.s). Samples of pig blood containing added *T. infestans* saliva shows a reduction in viscosity due to the disaggregation of erythrocytes (Figure 4). The rounded aspect of the erythrocyte disappears soon after the addition of this saliva, and it is possible that *T. infestans* saliva alters the blood's osmolarity, resulting in a flow of liquid into the interior of the erythrocyte. Complete disaggregation was observed in blood samples to which 50% of saliva had been added and, because the viscosity of this saliva is so low, it diffuses very easily in blood plasma.

The erythrocytic disaggregation of pig blood in response to the addition of *T. infestans* saliva was also detected by the Microplate Reader, as indicated in Figure 3. The addition of saliva produces an increase in absorption due to the separation of the *rouleaux* in the individual erythrocytes. Normal pig blood tends to aggregate and sediment, with a consequent decline in absorption. Pig plasma did not affect the study of the physical properties of aggregation because no absorption bands were present in the plasmatic protein at the wavelength of $\lambda = 655$ nm (Figure 3a and 3b).

The Microplate Reader also records the process of transformation that bovine erythrocytes undergo from a non-aggregated to an aggregated state, as indicated in the sequence of images in Figure 6. In Figure 5, note the increase in the inclination of the absorption curves (indicated by an arrow) as the fibrinogen concentration increased. In this case, the decline in the level of absorption was related to the sedimentation of the *rouleaux* that formed in an interval of 0 to100 seconds. The greater this decline, the higher the concentration of fibrinogen. Because bovine blood does not aggregate with the addition of up to 7.5 g/L of fibrinogen, the absorption, A, tends to approach the value of A for blood without added fibrinogen. With the addition of more than 10 g/L of fibrinogen, the inclination of the curve rises fairly sharply in response to the formation of *rouleaux*, and the sedimentation of these *rouleaux* results in sharp drop in A.

Acknowlegdments

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