In vitro study of rheological effects of propofol on human erythrocyte membrane

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Abstract
Research has demonstrated that anesthetics can affect hemodynamic properties of patients throughout surgery, since patients with normal laboratory values may need compensation after administration of anesthesia. In this work, we study the hemorheologic action of propofol \textit{in vitro} to assess its hemocompatibility and the possible effects on microcirculation. Propofol was used at different concentrations (2 to 20 $\mu$g/mL) similar to the ones reached during surgery to evaluate whether this anesthetic can modify the capacity of erythrocyte aggregation and viscoelasticity of red blood cells (RBCs). Results show that erythrocyte elastic modulus ($\mu$) increased by 20 $\mu$g/mL while membrane surface viscosity ($\eta_{m}$) diminished with the increment of propofol up to 5 $\mu$g/mL, being higher than the control for 10 and 20 $\mu$g/mL. The dynamic viscoelasticity is altered for the treated samples with propofol up to 5 $\mu$g/mL and aggregation capability ($C_{CA}$) of the treated RBCs increase in comparison to the control. Results suggest that propofol could produce alterations in the rheological behavior of erythrocyte membrane, which would exist at propofol concentrations of 2 - 5 $\mu$g/mL (near to those of steady-state).

Keywords: Propofol, hemorheology, anesthesia

1. Introduction

General anesthesia, either with inhalation or nonvolatile anesthetics, is known to affect the overall cardiovascular function as well as the microcirculatory hemodynamics \cite{1,2}. During the operation and postoperative period various hemorheological and hemostasiological alterations acquire clinical significance: (1) hyperreagibility of platelets with increased aggregation and adhesion tendency, (2) changes in fibrinogen, albumin and globulin concentrations, which affect viscosity and red cell aggregation, (3) impairment of red cell deformability, (4) increase in clotting factors, (5) disturbance of fibrinolysis characterized by diminution of plasmatic plasmin and increase in antiplasmin activity \cite{3,4}.

Propofol is being increasingly used for sedation in critically ill patients and for general anesthesia via continuous infusion because it allows prompt emergence with a low incidence of postoperative nausea and vomiting \cite{5,6}. In addition, it has recently been reported that propofol has a protective effect on the liver and intestinal microcirculation by increasing functional sinusoidal density and functional capillary density \cite{7}. However, it has been reported that propofol induces a slight, dose-dependent, echinocytic shape transformation of erythrocytes in vitro \cite{8} and erythrocyte deformability in an animal model \cite{9}. Furthermore, alterations in blood rheology under the influence of anesthesia may be responsible for the deterioration of tissue and organ perfusion related to anesthetic procedures \cite{10}. Recently, Kim et al. \cite{10} studied the time-related effect of propofol on red blood cell (RBC), and showed that this anesthetic had no direct effects on RBC deformability, aggregation, or morphology at clinical doses over a 4-hour incubation period. This was achieved using measurement techniques different from the ones commonly used in our laboratory.
We studied the *in vitro* effect of propofol at the plasma concentrations required for sedation and general anesthesia in order to evaluate whether this anesthetic can modify the capacity of erythrocyte aggregation and viscoelasticity of red blood cells. Measurements were carried out with an Erythrodeformeter [11,12] and by means of the digital analysis of microscopic images [13,14,15].

We studied the rheological parameters of the RBC membrane in a stationary regimen (DI: deformability index; $\eta_m$: superficial viscosity membrane; $\mu$: erythrocyte elastic modulus) and in a dynamic regimen in oscillations frequencies near the physiological range (phase shift at 0.5, 1 and 1.5 Hz), and we also analyzed the morphology of the cell aggregates by means of the isolated cell coefficient (C_{CA}) and the aggregation shape parameter (ASP).

2. Methods

2.1. Biological samples

Whole Blood samples were obtained from healthy donors without any known significant or pre-existing health problems (e.g. cardiovascular disease, respiratory disease, endocrine disease, haematological disorders or neoplasm etc.) and with normal hepatic, and renal function, coagulation and complete blood count. Blood samples were drawn by venipuncture and collected in sterile tubes containing EDTA as an anticoagulant. Collection and processing of samples were performed in duplicate at (22 ± 2) °C, according to the recommendations of the ICSH Guidelines on Blood Rheology [16]. The study was also performed in accordance with the Ethical Guidelines of Clinical Hemorheology and Microcirculation [17].

2.2. Sample treatment

Propofol (Propofol Gray® 10 mg/mL, DR. GRAY, Buenos Aires, Argentina) was diluted in physiological solution (PS) to obtain propofol concentrations of 0, 4, 6, 8, 10, 20 and 40 µg/mL. One mL of these solutions was added to one mL of blood, which resulted in final propofol concentration of 0, 2, 3, 4, 5, 10 and 20 µg/mL. The samples were incubated in chamber at 37°C for 30 min in constant agitation. After that time samples were centrifuged at 2500 rpm and washed three times with PS. Control sample was treated only with PS.

2.3. Study of the morphology of RBC aggregates

The study of the aggregate morphology was performed analysing the digital images obtained with transmission white light microscopy. Red Blood Cells (RBCs) were resuspended to 0.20% in autologous plasma placing 30μL of each suspension on an excavated slide for observation. The different propofol-RBC treatments were visualized and recorded with an inverted optical microscope (Union Tokyo Optical) using a 40x objective and a digital camera (Canon Power Shot A640 10.0 Mega Pixel) 9x zoom coupled with an adapter of 52mm. Three images were obtained for each sample.

2.3.1. Aggregate Shape Parameter

The method to study the morphology of RBC aggregates was through the determination of the shape parameter of the aggregate (ASP: Aggregate Shape Parameter). This dimensionless parameter that characterizes the morphology of the aggregates is defined by Eq. 1 [18]:

$$ASP = \frac{4\pi A}{P^2}$$

where $A$ is the projected area of the aggregate and $P$ is its perimeter. This parameter measures the circularity of the shape. A circle has maximum area/perimeter, giving an ASP = 1; consequently, the ASP values range from 0 to 1.
For this determination, images were processed with ImagePro software tracing the perimeter and projected area of each aggregate made up of 5 or more cells. The corresponding ASP values were calculated and the statistical analyses of the 3 images for each sample were carried out.

2.3.2. Individual Cell Coefficient

Individual Cell Coefficient (C_{CA} for its acronymic in Spanish) was defined as the difference between initial (Control RBC) and final (Propofol Treated RBC) individual cell (CA for its acronymic in Spanish) numbers related to the individual cell number of the control sample (Eq. 2).

\[
C_{CA} = \frac{C_{A_{initial}} - C_{A_{final}}}{C_{A_{initial}}}
\]

This coefficient varies between 0 (no differences in the aggregation before and after treatment) and 1 (complete aggregation after treatment)

2.4. Evaluation of erythrocyte viscoelasticity

For the determination of stationary and dynamic viscoelastic parameters of RBCs, we used the Erythrodeformeter [11,12], which was developed in our laboratory and is based on the laser diffractometry technique. This instrument measures the ability of RBCs to change from discoid to ellipsoidal shape, when subjected to a shear stress. The theoretical analysis of RBC deformability based on ellipsoidal cell shapes under shear stress has been detailed before [13,14,15]. This analysis is used to determine the following membrane viscoelastic parameters:

- \( \mu \): Elastic Modulus of Erythrocyte
- \( \eta_m \): Surface Viscosity of Erythrocyte Membrane
- DI: Erythrocyte Deformability Index
- \( \delta \): Phase-shift between applied shear stress and erythrocyte response at 0.5, 1 and 1.5 Hz.

The hematocrit was adjusted to 40 % by resuspending the RBCs in a calculated autologous plasma volume. 100 \( \mu \)L of these suspension for each sample (control or treated) were poured in 4.5 mL of a solution of polyvinyl-pyrrolidone (PVP, PVP360\( ^\circ \), Sigma) at 5% (w/v) in phosphate buffer solution (PBS); (viscosity = (22 ± 0.5) cp, pH = (7.4 ± 0.05) and osmolarity = (295 ± 8) mOsmol/kg at (25.0 ± 0.5) °C).

2.7 Statistical Analysis

To study the effect on the hemorheological parameters of the RBC treatment with different concentrations of propofol, we used GraphPad 4.0 software.

3. Results

The flow in small vessels is sensitive to the size and shape of the aggregates. Besides, aggregate structures affect microvascular resistance to blood flow. Alterations in aggregate morphology induce complications in microcirculation. Accordingly, we decided to study the effect of propofol on the morphology of RBCs aggregates at different concentrations of this anesthetic.

The propofol final concentrations were chosen taking into account the ones reached during a surgery in the steady state (2 to 4 \( \mu \)g/mL). In Fig. 1 the visual observation of the RBCs aggregates is shown. At plain sight it can be seen that the number of free cells is much lower for all the treated samples compared to the control. This observation is consistent with the \( C_{CA} \) values calculated for these samples (Table 1). On the other hand, no significant changes in ASP values were observed between the groups (Table 1). This result is also in agreement with the shapes of the aggregates observed at a glance on the images (Fig. 1).

Since the formation and rupture of the aggregates depends, among other things, on the elastic
properties of the membrane, we studied the behavior of RBCs in the samples treated and non-treated in an Erythrodeformeter so as to know the values of DI, $\mu_m$ and $\eta_m$ and their behavior at oscillation frequencies within the physiological range ($\delta$).

Fig. 1. Microscopic images of red blood cell aggregates. Red Blood Cells (RBCs) were resuspended to 0.20% in autologous plasma as depicted in materials and methods. A representative image is shown for each treatment.

Table 1
Stationary viscoelastic parameters, ASP and $C_{CA}$ determinations

<table>
<thead>
<tr>
<th>Propofol</th>
<th>Control</th>
<th>2 µg/µL</th>
<th>3 µg/µL</th>
<th>4 µg/µL</th>
<th>5 µg/µL</th>
<th>10 µg/µL</th>
<th>20 µg/µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI</td>
<td>0.665 ± 0.007</td>
<td>0.670 ± 0.007</td>
<td>0.648 ± 0.01</td>
<td>0.657 ± 0.01</td>
<td>0.658 ± 0.008</td>
<td>0.666 ± 0.006</td>
<td>0.655 ± 0.006</td>
</tr>
<tr>
<td>$\mu_m$ ($10^{-3}$ dyn/cm)</td>
<td>4.8 ± 0.5</td>
<td>4.6 ± 0.6</td>
<td>4.3 ± 0.1</td>
<td>5.1 ± 1.1</td>
<td>5.4 ± 0.9</td>
<td>5.6 ± 0.9</td>
<td>9.8 ± 0.5**</td>
</tr>
<tr>
<td>$\eta_m$ ($10^{-4}$ dyn-s/cm)</td>
<td>2.0 ± 0.2</td>
<td>2.32 ± 0.3</td>
<td>1.7 ± 0.7</td>
<td>1.6 ± 0.7</td>
<td>1.9 ± 0.7</td>
<td>3.8 ± 0.7*</td>
<td>3.3 ± 0.1***</td>
</tr>
<tr>
<td>ASP</td>
<td>0.64 ± 0.02</td>
<td>0.67 ± 0.02</td>
<td>0.69 ± 0.02</td>
<td>0.69 ± 0.02</td>
<td>0.71 ± 0.02</td>
<td>0.70 ± 0.01</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>$C_{CA}$</td>
<td>0</td>
<td>0.9 ± 0.2**</td>
<td>0.7 ± 0.2*</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.2*</td>
<td>0.5 ± 0.2*</td>
<td>1.0 ± 0.2**</td>
</tr>
</tbody>
</table>

Unpaired Student t-test was performed between control and treated samples; *** p<0.001; ** p<0.01; * p<0.05
As depicted in Table 1, no significant changes in the stationary viscoelastic parameter DI was observed between the groups, but results showed that μ increased for 20 µg/mL while ηm decreased with the increment of propofol up to 5 µg/mL, being statistically higher than the control for 10 and 20 µg/mL.

Phase shift was determined at oscillation frequencies within the physiological range (0.5, 1 and 1.5 Hz). The phase shift for 1.5 Hz decreased for the treated samples up to 5 µg/mL propofol when compared to the non treated ones in a concentration dependent manner. There were no significant changes for the higher concentrations 10 and 20 µg/mL (Fig. 2).

![Phase shift vs. Propofol concentration](image)

**Fig.2.** Phase shift vs. Propofol concentration. Phase shift was determined at oscillation frequencies within the physiological range (0.5, 1 and 1.5 Hz). A typical result is shown.

### 4. Discussion

In this work we studied the effect of different concentrations of propofol on the viscoelastic parameters of red blood cells in vitro.

In spite of the results recently published by Kim et al. [10], this study suggests that propofol may produce alterations in the rheological behaviour of erythrocyte membranes, at propofol concentrations of 2, 3, 4 and 5 µg/mL (near to those of steady-state), lower than the ones previously published in the bibliography. The discrepancy observed between these two works could be due to differences in the sensitivity of the test carried out and because other viscoelastic parameters were calculated.

Under our assay conditions, we observed that propofol affected C_{CA}, this coefficient being higher for the treated samples when compared to the control. This might be indicating that propofol treated RBCs tend to aggregate regardless of the concentration of propofol used. The higher or lower tendency of RBCs to aggregate depends on the factor balance that helps or hinders aggregation. Between aggregation inhibitors can the following be mentioned: (1) electrostatic repulsion between RBCs caused by the negative charge on the erythrocyte surface, (2) membrane elasticity, since aggregation involves some degree of deformation of the erythrocyte and (3) the existence of flow and shear rate which tends to separate the aggregated erythrocytes. Therefore, propofol could be affecting the membrane surface charge, resulting in an increased number of cells aggregated.

Furthermore, both ηm (membrane viscosity) and μ (elastic modulus) were altered. ηm diminished while propofol concentration increased up to 5 µg/mL, indicating that this anesthetic could be acting not only over glycocalyx charge but also on the lipid bilayer. In fact, it has been reported that propofol presents high fat solubility, and that this anesthetic could be sequestered in red blood cells on rabbits [19]. This ability of propofol to intercalate and overpass the lipid bilayer could be the reason why there is a ηm decrease and consequently an increase in membrane rigidity. Moreover, μ was increased for the higher propofol concentration assayed. This may indicate that this anesthetic would act not only at membrane level but also on cytoskeletal proteins by altering cell elasticity at high concentration.

Finally, we observed that there were differences in the phase shift between the control and the treated samples. A phase shift of zero means that the sample behaves as a solid, and a phase shift of π/2 means
that the sample is an ideal fluid.

The results presented here show that phase shift diminished as propofol concentration increased up to 5 μg/mL, meaning that at higher propofol concentrations RBCs behave more like a solid than the control sample, indicating a membrane rigidification.

All these results could indicate that propofol could be in fact altering RBCs viscoelasticity and aggregation capability. Although the changes in the viscoelastic parameters presented here are slight, these variations could be significant in pathologies such as diabetes and hypertension, where an alteration in the membrane viscoelasticity has been reported.

References