In vitro alteration on erythrocytes mechanical properties by propofol, remifentanil and vecuronium

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ABSTRACT

Several studies report flow disturbance and microcirculation disorders upon anesthesia treatment. These alterations are often related to blood rheology changes. In this work, it was attempted to make a detailed description of the alterations in erythrocyte mechanical properties by the action of propofol, remifentanil, and vecuronium. For this, an *in vitro* study was performed on red blood cell samples from healthy donors incubated with solutions of propofol (4 μg/ml whole blood), remifentanil (10 ng/ml plasma), and vecuronium (0.15 μg/ml plasma). Erythrocyte viscoelastic parameters were determined by octuplicate using a Reómetro Eritrocitario. Also, a Wilcoxon signed rank-test with Yates correction for continuity was performed to analyze the overall alteration in the mechanical properties of erythrocytes. Statistical analysis showed that the three studied anesthetics changed the erythrocyte mechanical properties at different parts of the membrane. These results would imply an interaction of these anesthetics with the erythrocyte membrane. Finally, this could conduce to alterations in microcirculation.

KEYWORDS

Propofol; remifentanil; vecuronium; erythrocyte viscoelasticity; hemorheology

ABBREVIATIONS

RBC, red blood cell; C, control sample; P, propofol; R, remifentanil; V, vecuronium; μ, elastic modulus of membrane; η_m, surface viscosity of membrane; DI, erythrocyte deformability index; G′, storage modulus (or dynamic elastic modulus); G″, viscous modulus (or loss modulus).

1. INTRODUCTION

Drugs used during anesthesia could induce microvascular flow disturbance due to their systemic cardiovascular actions and the direct effect on microcirculation. This disturbance generally occurs by hemorheological changes related to an increase in platelet aggregation, changes in the viscosity and deformability of erythrocytes (red blood cells, RBCs), an increase in coagulation factors, and a decrease in fibrinolysis (Pandharipande et al., 2011; Caglayan et al., 2006; Alet et al., 2012, 2015). On the other hand, patients with hematological and vascular diseases (diabetes, hypertension, dyslipidemia, etc.) show hemorheological alterations, particularly in the blood viscosity, mechanical properties of the erythrocyte membrane, and erythrocyte aggregation (Alet et al., 2001; Delannoy et al., 2014; Gyawalli et al., 2014; Riquelme et al., 2003). Then, in these patients, it is of interest to detect possible previous patho-hemorheological and patho–hemostasiological alterations to take preventive actions before surgery, obtaining the maximum benefit for the patient (How, 1996; Popel and Johnson, 2005).

Gyawali et al. (2014) showed that propofol has cardiovascular effects such as a decrease in peripheral vascular resistance or jugular venous oxygen saturation, and induced hemolysis among other consequences. Also, it can cause the so-called propofol infusion syndrome, a rare but lethal condition (Cremer et al., 2001).

Moreover, results obtained from a previous study of the effect of propofol on aggregation of RBCs from healthy donors (Alet et al., 2012), showed that *in vitro* propofol treatment could produce slight alterations to the erythrocyte membrane at a concentration of 4 μg/mL (steady-state). Furthermore, previous research showed that propofol treatment alters the dynamic viscoelastic parameters (Batista da Silva et al., 2017). These results also suggest that propofol could alter RBCs aggregation and cell elasticity.

Until now, there are no detailed studies about the action of remifentanil and vecuronium on the erythrocyte membrane. Since these drugs present certain lipophilicity because of their structure, more research is needed to know its possible hemorheological effects.

Therefore, this work aimed to make a detailed description of the alterations in the erythrocyte mechanical properties by the propofol treatment and to inquire into the possible hemorheological effects of remifentanil and vecuronium.

2. MATERIALS AND METHODS

2.1. Biological samples

Human blood samples from 5 healthy male donors (23 to 33 years old, no intake of any medication for the last 7 days, non-smoker, normotensive, with normal hemogram and without any known-significant or pre-existing health problems) were collected by venipuncture in sterile vials containing $EDTAK_3$ as anticoagulant. The blood sample hematocrit was adjusted at 40% with autologous plasma. Collection and processing of samples were performed within 4 hours from extraction time (Baskurt et al., 2007).

The study was approved by the Bioethics Committee of the Facultad de Ciencias Bioquímicas y Farmacéuticas of the Universidad Nacional de Rosario (Res. Nº 347/2013 on June 18th, 2013) and all donors signed the informed consent. Complete clinical history and physical examination, including standard laboratory tests, were performed on each donor.

2.2. Anesthetics work solution preparation

Propofol commercial suspension (P) (Propofol Gray® 10 mg/mL, DR. GRAY, Buenos Aires, Argentina) was diluted in normal saline solution (0.90% w/v of NaCl, 308 mOsm/L, Laboratorio de Especialidades Medicinales, Rosario, Argentina) to obtain propofol concentration of 8 μg/mL.

Remifentanil (R) (Remifentanilo Kabi® 5 mg, lyophilized powder, Fresenius Kabi, Buenos Aires, Argentina) was diluted in normal saline solution to obtain remifentanil concentration of 12 ng/mL (corresponding to 20 ng/mL plasma).

Vecuronium (V) (Vecuronio Northia® 10 mg, lyophilized powder, Northia, Buenos Aires, Argentina) was diluted in normal saline solution to obtain vecuronium concentration of 0.18 μg/mL (corresponding to 0.30 μg/mL plasma).

2.3. Sample treatment with intravenous anesthetics

Previously, an experimental dose-response study was carried out for propofol to determine the suitable concentrations for the present study (Alet et al., 2012). Also, the anesthetic concentrations used in this study corresponded to the physiological concentrations employed during anesthesia (steady-state concentrations), which are depicted in the specific literature (Miller et al., 2000). Then, one volume of the corresponding work solution was added to one volume of blood sample, which resulted in final concentrations: P at 4 μg/mL, R at 6 ng/mL (corresponding to 10 ng/mL plasma) and V at 0.09 μg/mL (corresponding to 0.15 μg/mL plasma) (Miller et al, 2000). The samples were incubated at 37ºC for 30 min in constant slow stirring. After 15 min of incubation with R, another dose was added because of its short lifetime. Since red blood cells do not metabolize P and V, an additional dose of these two anesthetics is not necessary, and they remain at the initial concentration during incubation.

Samples were then centrifuged at 1800 g and washed three times with normal saline solution. The RBC packages were reserved for further analysis. Control sample (C) was considered as the RBC incubated only with normal saline solution.

2.4. Mechanical properties of Erythrocyte

Measurements were carried by octuplicate using the Reómetro Eritrocitario (AR91467B1, Riquelme et al., 2013), which was developed in our laboratory (IFIR, CONICET-UNR) (Riquelme et al., 2018; Castellini et al., 2018, 2019). This instrument is based on laser diffractometric technique and measures the time deformation of RBCs subjected to steady and oscillatory shear stresses (Bessis and Mohandas, 1975). Several authors have validated this technique, and the measurements and parameter determinations are similar to the described by Rasia et al. (1986, 1995) and Riquelme et al. (1986, 1997, 2003, 2006). Briefly, in this instrument, a thin layer of RBC suspension is placed between two parallel concentric disks, an upper static disk and a lower rotating one. In the steady regime, the lower disk rotates at a constant speed (64 rpm, giving the shear rate of approximately 1300 s^{-1} corresponding to a shear stress of about 28 Pa). In the dynamic regime, it

moves at a sinusoidal oscillating speed at three pre-established frequencies (0.5, 1, and 1.5 Hz). A laser beam perpendicularly traverses the suspension of sheared RBCs, producing a diffraction pattern. This pattern is circular when the RBCs are static (immobile) and becomes elliptical when the cells undergo shear stress.

Photometric readings performed along both axes of the diffraction pattern are used to determine the RBC rheological parameters, which are averaged over several millions of cells. The theoretical analyses of RBC deformability are based on ellipsoidal cell shapes under shear stress. They are explained in detail in the references (Riquelme et al., 1986, 2005, 2006, 2018; Castellini et al., 2018, 2019; Bessis and Mohandas, 1975). These analyses were used to determine the steady and dynamic viscoelastic parameters of RBC to know if there exist any intrinsic variations in membrane elasticity by the action of anesthetics.

The steady viscoelastic parameters of the erythrocyte membrane obtained in the steady regime were:

DI: erythrocyte deformability index

μ: elastic modulus of RBC membrane

η_m: surface viscosity of RBC membrane

where DI was calculated as:

$$DI = \frac{A_1 - A_3}{A_1 + A_3}$$

being A_1 and A_3 the readings taken along the major and minor axes, respectively, of the elliptical pattern in the steady regime.

The elastic modulus (μ) and the membrane surface viscosity (η_m) were calculated by numerical processes from creep and relaxation curves. These parameters are related by:

$$\eta_m = t_c \cdot \mu$$

where t_c is the characteristic retardation time (calculated by fitting an exponential function with the recovery curve).

The dynamic viscoelastic parameters of RBC were obtained in a dynamic regime. In this regime, the strain is pulsating, similar to what occurs in the physiological state by the cardiac cycle. The oscillation frequency used were 0.5, 1, and 1.5 Hz corresponding to bradycardia (30 cycles per minute), normal cardiac frequency (60 cycles per minute), and tachycardia (90 cycles per minute), respectively. In this case, light intensity variations along the major axis of the diffraction pattern were recorded, and these data were used to calculate the phase shift (δ) between applied stress and RBC response. Then, the following dynamic viscoelastic parameters were calculated for each sample:

G′: storage modulus (or dynamic elastic modulus)

G″: viscous modulus (or loss modulus)

G′ and G" are the real and imaginary components of the complex elastic modulus, which can be expressed as:

$$G^* = G' + i\,G''$$

These parameters can be expressed as follows:

$$G' = \frac{\sigma_0}{\gamma_0}\cos(\delta)$$

$$G'' = \frac{\sigma_0}{\gamma_0}\sin(\delta)$$

where $\frac{\sigma_0}{\gamma_0}$ is the steady elastic modulus of the erythrocyte membrane, μ. These parameters can be satisfactory used to describe the viscoelastic characteristics of the erythrocyte membranes as shown elsewhere (Riquelme et al., 1986, 2003, 2005, 2006; Batista da Silva et al., 2017; Ponce de Leon et al., 2020)

Steady and dynamic determination were carried out subsequently. For these measurements, the hematocrit of each sample was adjusted to 40 % by suspending the RBCs in autologous plasma. Finally, 100 μL of each sample (control or treated) were poured in 4 mL of a solution of polyvinyl-pyrrolidone (PVP, PVP360®, Sigma) at 5% (w/v) in phosphate buffer solution (viscosity = (22 ± 0.5) cp, pH = (7.4 ± 0.05), osmolality = (295 ± 8) mOsmol/kg at (25.0 ± 0.5)°C).

2.5. Statistical Analysis

Data were obtained from samples from 5 healthy donors, as stated before. For each donor, one tube per treatment was prepared to evaluate the effect of C, P, R and V. Then, 8 curves were recorded to determine μ, t_c, DI and δ values, yielding 8 replicates values of each parameter for each donor and each treatment. After that, matrixes were performed to determine η_m, G' and G" values at the different frequencies, yielding 64 values of each parameter for each donor and each treatment.

For each parameter analyzed, the obtained data were grouped by each donor and by treatment (C, P, R, V). Finally, a One-way ANOVA test was performed to assure data homoscedasticity for the same treatment between donors, being the null hypothesis that all data belong to the same population. In the case that the null hypothesis was rejected, a Tukey test was performed to find the values that have a really significant difference (RSD), expressed by:

$$RSD = q_{\alpha,k,n-k}\sqrt{\frac{CM_d}{N}}$$

where $q_{\alpha,k,n-k}$ was obtained from the Tukey tables, k is the number of data, $n-k$ is the freedom degree found in the One-way ANOVA test, CM_d is the sample variance, and N is the minimum between the data from the compared samples.

Each donor data set is rejected if the RSD is greater than its media. Finally, those groups of data (donor) that belong to the same population for the same parameter and treatment can be found. Then, the data belonging to the same population for the same treatment and parameter are selected and grouped. Subsequently, they were compared to the Control group, which has been subjected to the same kind of analysis. A Student's t-test was performed between each treated sample and the corresponding control to asses if there is or not a significant difference (p-value < 0.05). This procedure was employed for μ, η_m, DI, G' and G" parameters.

On the other hand, a Wilcoxon signed-rank test with the Yates's continuity correction for non-parametric variables was performed to analyze the overall alteration in the viscoelastic parameters by the anesthetic action (null hypothesis was that there is no significant difference between control

and treatment). If the null hypothesis is rejected, there is enough evidence to conclude that the treatment could be affecting the membrane dynamics. All statistical analyses were executed with RStudio software.

3. RESULTS

Table 1 shows the mean values of steady viscoelastic parameters (μ, η_m and DI) obtained from control and RBCs treated with the anesthetics. Analysis of results from Table 1 shows that membrane surface viscosity was significantly lower in all the treated samples when compared to C (P and R is $p < 0.01$; V is $p < 0.0001$).

Table 1: *Steady viscoelastic parameters of erythrocyte. Data are given as (mean ± SD).*

RBC sample	η_m 10^{-7} N.s/m	μ 10^{-6} N/m	DI
C	2.44 ± 0.41	4.94 ± 0.31	0.61 ± 0.05
P	2.04 ± 0.07**	4.86 ± 0.15	0.59 ± 0.02*
R	2.10 ± 0.06**	4.88 ± 0.24	0.60 ± 0.04
V	1.89 ± 0.04****	4.90 ± 0.15	0.61 ± 0.05

C, control; **P**, treated with propofol 4 μg/ml whole blood; **R**, treated with remifentanil 10 ng/ml plasma; **V**, treated with vecuronium 0.15 μg/ml plasma; η_m, membrane surface viscosity; **μ**, elastic modulus; **DI**, deformability index. "*" denotes p-value by Student's t-test: *($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$).

The elastics modulus μ remained constant with the anesthetic treatments. Nonetheless, the deformability index (DI) was significantly lower for P treatment when compared to C ($p < 0.05$). The values of storage modulus (G') and viscous modulus (G") calculated for each oscillation frequency are presented in Table 2.

Table 2: *Dynamic viscoelastic parameters of erythrocyte. Values are presented as (mean ± SD) Data are given as (mean ± SD).*

RBC Sample	G' 10^{-6} N/m			G" 10^{-6} N/m		
	0.5 Hz	**1.0 Hz**	**1.5 Hz**	**0.5 Hz**	**1.0 Hz**	**1.5 Hz**
C	4.79±0.23	3.88±0.18	2.05±0.11	1.22±0.06	3.00±0.15	4.53±0.20
P	4.70±0.09	3.85±0.06	2.03±0.03	1.50±0.08****	2.89±0.20	4.43±0.13
R	4.71±0.24	4.02±0.22*	1.91±0.12**	1.21±0.07	2.54±0.19****	4.45±0.21
V	4.71±0.12	4.05±0.07***	1.91±0.09***	1.32±0.04****	3.02±0.12	4.54±0.09

C, control; **P**, treated with propofol 4 μg/ml whole blood; **R**, treated with remifentanil 10 ng/ml plasma; **V**, treated with vecuronium 0.15 μg/ml plasma; **G'** storage modulus; **G"** loss modulus. "*" denotes p-value by Student's t-test: *($p<0.05$), **($p<0.01$), ***($p<0.001$), ****($p<0.0001$).

When comparing the values obtained for each anesthetic treatment with the control, different kinds of alterations in the erythrocyte mechanical properties for the three oscillation frequencies were observed. In particular, R and V induced significant increments in G' at 1.0 Hz ($p < 0.05$ and $p < 0.001$), while this parameter was significant diminished for 1.5 Hz ($p < 0.01$ and $p < 0.001$), indicating in both cases an interaction with the cytoskeleton proteins. Results from P treatment showed a significant increase of G" at the lower frequency ($p < 0.0001$). Furthermore, the treatment with R induced a significant decrease of G" at 1.0 Hz ($p < 0.0001$) and the treatment with V a significant increment at 0.5 Hz ($p < 0.0001$). No significant changes were observed for G" at 1.5 Hz for all treatments.

The table 3 shows the results from the statistical assay, performed considering all the mechanical parameters together. These results indicated that for R and V, the RBC mechanical parameters were significantly altered in all groups when compared to control ($p=0.0098$).

Table 3: *Results of Wilcoxon signed rank-test with Yates's correction for non-parametric variables. Mean score comparison.*

RBC sample	p-value	H0=does not affect (significance<0.05)
P	0.1587	Accepts
R	0.0098	Rejects
V	0.0098	Rejects

P, treated with propofol 4 μg/ml whole blood; **R**, treated with remifentanil 10 ng/ml plasma; **V**, treated with vecuronium 0.15 μg/ml plasma; **H0**, null hypothesis.

DISCUSSION

Several works have shown that propofol treatment at steady-state conditions could be altering RBCs at different parts of the erythrocyte membrane (cell surface, intramembrane and intracellular) (Mazoit et al., 1999; Bahri et al., 2007). In this work, results show that P, R and V alter the rheological characteristics of human RBCs. Our observation suggests that these drugs have different kinds of interactions with membrane structure and intracellular dynamics.

Present results show a significant alteration in both η_m and DI for P treatment, and agrees with previous reports suggesting an interaction with the lipid bilayer (Hansen et al., 2013). Also, the R and V treatments decreased the η_m of the RBCs, indicating that they would also be interacting with the lipid bilayer. The explanation of these results in the case of propofol could be because it can intercalate in the lipid bilayer and behave similarly to cholesterol (Hansen et al., 2013). This behavior is because of the lipophilic characteristics of propofol. Furthermore, the intercalation of propofol induces a disorder in the lipid bilayer and modifies its microviscosity (Hansen et al., 2013). Since R and V also have a similar lipophilic behavior as P, it is expected that both anesthetics also modify η_m. On the other hand, increments in surface membrane viscosity have been associated with the stiffening of the RBC (Liu et al., 2007), rendering in a probable microcapillary occlusion. In this study, anesthetics treatment induced a lower η_m than the C, resulting probably in

the red blood cells becoming softer. This cellular softening could decrease the possibility of microcapillary occlusion but also weaken the membrane to resist the continuous changes in blood flow.

Mazoit et al. (1999) have shown that anesthetics (principally propofol) can insert in the lipid bilayer and translocate to the cytoplasm. These interactions could be the explanation for the alteration observed in the dynamic viscoelastic parameters, representatives of the membrane elasticity (G'), and the resistance to flow (G"). A lower value of G' could imply a cytoskeleton alteration leading to a membrane softening, similar to what happens in an elastic spring. This is the case for R and V at 1.5 Hz. On the other hand, a higher G' value would be indicating the opposite behavior, as seen for R and V but at 1.0 Hz. Furthermore, a higher value of G" could indicate that the anesthetic alters the lipid bilayer viscosity, leading to an increment of the internal frictional force (P and V at 0.5 Hz), and a lower G" value would be indicative of the opposite effect (R at 1.0 Hz). Consequently, when the molecule under assay interacts closely with all parts of the human erythrocyte membrane (glycocalyx, lipid bilayer, cytoskeleton, and anchor proteins), it could alter these dynamic viscoelastic parameters.

Summarizing, in this work non-significant variations with the anesthetic *in vitro* treatments were observed for G' at 0.5 Hz (bradycardia). Nevertheless, at 1.0 Hz (normal cardiac frequency) and 1.5 Hz (tachycardia), G' shows significant changes, which have different behavior even for the same anesthetic. An opposite behavior is observed for G" with the oscillatory frequencies. These results would agree with the fact that each anesthetic would interact with different membrane proteins, which are differently related to viscoelastic properties (Noguchi, 2010; Puig-De-Morales-Marinkovic et al., 2007). Still, there is a lot of work in this field to unravel the molecular mechanism behind this behavior. Future studies are needed to clarify the anesthetic interaction at the molecular level of the erythrocyte membrane.

Finally, when analyzing the general effect of these anesthetics, the current evidence would indicate that V and R significantly affect the mechanical behavior of erythrocytes. In contrast, there would

be insufficient evidence to indicate a significant alteration in the general mechanical properties of erythrocytes due to P treatment. It is worthily to note that the propofol employed in this work is formulated in a lipid emulsion.

CONCLUSIONS

The steady and dynamic parameters (at 0.5, 1 and 1.5 Hz) of the erythrocyte membrane would be altered in different ways by *in vitro* treatment with propofol, remifentanil or vecuronium. Significant alterations of erythrocyte dynamics were observed by remifentanil and vecuronium treatment, when the mechanical behavior of erythrocytes is considered as a whole. These results would imply a close interaction of these anesthetics with the erythrocyte membrane, which could conduce to alterations in microcirculation.

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