Effects of Propentofylline on the Micromechanical Properties of Red Blood Cells

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ABSTRACT


The hemorheological effects of the xanthine derivative propentofylline (HWA 285) were investigated in vitro and ex vivo. The following micromechanical methods were used: (1) Nuclepore filtration test; (2) Filtrometer MF 4 [Teitel, 1977]; (3) Singlepore Erythrocyte Rigidometer (SER) [Kiesewetter et al., 1982b] (4) Mini-Aggregometer [Schmid-Schönbein et al., 1973a]. A protective effect of HWA 285, starting at a dose of 10^-5 mol/liter, against red blood cell (RBC) rigidification, induced by lactacidosis (pH 6.9) in the Nuclepore filtration test could be demonstrated. RBC deformability was shown to decrease in adjuvant arthritis (AA) in rats. We tested HWA 285 (1, 5.01, 10.0, 15.8, 31.5, and 50.0 mg/kg p.o. for 21 days) in AA rats for its effect on hemorheology. The drug neither had an effect on inflammation with respect to hematological parameters and plasma proteins nor caused a reduction in the increased RBC aggregation in AA rats. However, we could show with the first three methods that HWA 285 has a significant effect on improvement of RBC deformability.

Key words: hemorheology, propentofylline, red blood cell deformability, metabolic stress

INTRODUCTION

The fluidity of blood is determined by the micromechanical properties of its corpuscular as well as its plasmatic components and by the integrity of the vessel wall. Disorders in its rheological abilities lead to diminished perfusion of the microcirculation and cause a decrease

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in its nutritive function [Schmid-Schönbein, 1977]. The role of various factors in disturbed blood flow is the main subject of hemorheological research. The extent of blood fluidity and coagulation disorders seems to reflect the degree of microcirculatory disturbances in different diseases irrespective of their etiology. Changes in the mechanical and chemical characteristics of the red blood cells decrease the ability of the cells to adapt to flow. Local increase of lactic acid (e.g., stagnant anoxia) induces RBC rigidity [von Ardenne, 1978; Dintenfass, 1971], which may cause disorders in the microcirculatory blood flow.

Recent investigations have shown that cerebral blood flow is influenced to a great extent by hemorheological factors [Schmid-Schönbein, 1983]. More than 40% of patients with cerebrovascular disease (CVD) exhibit increased blood viscosity owing to decreased RBC deformability and increased RBC aggregation [Ott, 1983].

Investigations of HWA 285 on regional blood flow showed that the drug increases blood flow in the heart, brain, and skeletal muscle [Hudlicka et al., 1981]. Furthermore, the drug alleviates the development of cerebral edema by bilateral carotid ligation in gerbils [Mrsulia et al., 1983] as well as the energy metabolism in nitrogen-breathing rats [Stefanovich and Nagata, 1983b]. HWA 285 is known to have a direct effect through interaction with adenosine [Porsche, 1982; Stefanovich, 1983; Stefanovich and Nagata, 1983a] and to exert a modulative effect on the brain mitochondria, depending on their functional status, by increased phosphorylation [Porsche and Stefanovich, 1982].

It was, therefore, interesting to study the hemorheological effect of HWA 285. To simulate the pathophysiological conditions of diminished RBC fluidity, we have altered in vitro the rheological properties of RBC's by decreasing pH values (pH 6.9) with lactic acid [Seiffge, 1980]. Furthermore, it has been shown that RBC deformability is reduced in adjuvant arthritis in rats [Seiffge and Kremer, 1983]. This inflammatory disease served as an in vivo model. The drug was administered to the animals for 21 days, and some ex vivo hemorheological tests were performed.

METHODS
Preparation of Blood

The studies were carried out with blood from male dogs (beagle) and rats (Wistar). Venous blood samples from dogs were anticoagulated with sodium heparin (40 USP units/ml). Blood samples were taken from rats by means of heart puncture under light ether narcosis using sodium heparin (143 Vacutainer, USA). Hematological (Coulter Counter, model ZF) and biochemical measurements were carried out with fresh blood samples: 40% with autologous plasma for viscometry and rheoscopy, and 10% for filtration measurements. For this purpose blood was centrifuged for 7 min at 300 g at room temperature (20–22°C), the plasma and buffy coat carefully removed, and the hematocrit adjusted with a phosphate buffer solution (PBS: 29 g Na₂HPO₄ × 12 H₂O, 3 g KH₂PO₄/liter + 0.25% human albumin, pH 7.3, prefiltered through a 0.2-μm membrane). The plasma proteins were determined by membrane electrophoresis or by biuret test. Lactate values were estimated using the Lactate Analyzer 640 (Bio. Electronics, Roche).

Lactacidosis

Artificial plasma lactacidosis with a pH value of 6.9 was achieved by adding 1 ml lactic acid (10%) to 10 ml of the prefiltered plasma 5 min before resuspending the RBC.

Adjuvant Arthritis

Adjuvant arthritis was induced in male Lewis rats, body weight 200–300 g, by a subcutaneous injection of 0.1 ml Mycobacterium butyricum suspension (0.5%) in heavy paraffin oil into the plantar surface of the left hind paw. Paw volumes were recorded by means of a water displacement plethysmometer (2060, Rhema Labortechnik, Hofheim, FRG) [New-
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bould, 1963; Pearson, 1965]. Noninjected rats served as controls. The animals were fed commercial pellets (Altromin 1214) and allowed to drink water ad libitum. Measurements were carried out on the 21st day of the disease [Seiffge and Kremer, 1983].

**Erythrocyte Filtrability**

1) RBC filtrability was determined in the filtration equipment according to Reid et al. [1976], using the method of Schmid-Schönbein et al. [1973b]. RBC flow rate was measured by testing the ability of RBC’s to pass through a filter membrane (Nuclepore Corp.) from a batch with defined pore width (3 μm), length (10 μm), and density (4 × 10^5 pores per cm²) at a constant pressure gradient (Δp = 50 mm H₂O). The flow rate of each suspension (ml/min) was determined and the relative flow rate \( V_{rel} = \frac{V_{susp}}{V_{buffer}} \) was taken as a quantitative measurement of RBC filtrability. Only those batches of filters that showed a standard deviation of less than 10% were used for experiments. This was determined by using standard blood samples in a pretest [Seiffge, 1980].

2) The microcomputer-controlled Filtrometer MF 4 (Messrs. Myrenne, Roetgen, FRG) measures the flow curve of 2 ml of a RBC suspension (Hct 10%) through a filter membrane (Nuclepore Corp., pore diameter 3 μm) [Kiesewetter et al., 1982b; Teitel, 1977]. The measuring tube is U-shaped; RBC’s are placed in one side and the filter is inserted at the collateral side of the U-shaped tube. The decreasing pressure gradient initially comes to Δp = 30 mm H₂O. The initial slope (Init. sl.) after 10% of the solution has passed through the filter and the remaining volume (Rem. vol.) after flow has stopped are calculated.

3) The passage times of single erythrocytes through a single pore in a synthetic membrane (Makrofol N) are determined in the Singlepore Erythrocyte Rigidometer (SER) [Kiesewetter et al., 1982b]. The pore in the membrane represents a measuring capillary with preselected diameter (here 3.5 μm for rat blood) and length (30 μm). At a constant driving pressure of 70 Pa (= 7 mm H₂O), the passages of the RBC’s are measured by an electrical data detection device [Roggenkamp et al., 1983]. For each experiment, a microprocessor unit calculates the mean passage time (tₘ in msec) of 200 RBC.

**Erythrocyte Aggregation**

The erythrocyte aggregation was determined by a newly developed mini-aggregometer [Kiesewetter et al., 1982a], which is based on a method previously described [Schmid-Schönbein et al., 1973a]. Briefly, a transparent measuring chamber (cone/plate configuration) is trans-illuminated by infrared light. The intensity of the transmitted light, which is modified by the aggregation process after flow has stopped, is recorded within a definite period (10 sec) and converted into a dimensionless quantity. The microprocessor-controlled mini-aggregometer measures the mean extent of RBC aggregation (MEA).

**Plasma Viscosity**

The plasma viscosity was measured with the help of a Coulter Harkness Viscometer® at controlled temperature (37°C).

**Reagents**

The compound propentofylline (1-[5′-oxohexyl]-3-methyl-7-propyl-xanthine, HWA 285)¹ was synthesized as described elsewhere [Thorpe, 1982]. All other chemicals used were reagent grade or better. For in vitro investigations, the drug was added to the blood at different doses 30 min before in vitro testing, or administered orally at different doses for 21 days to the adjuvant arthritis (AA) rats (ex vivo); the same volume of physiologic NaCl was added to the control.

¹Synthesized by Hoechst AG Werk Albert, Wiesbaden (FRG).
Statistical Evaluation

The results obtained in the studies were statistically evaluated by means of the Student’s t-test (P < 0.05).

RESULTS

The drug HWA 285 shows in vitro a significant improvement of erythrocyte filtration rate in the Nuclepore filtration test under artificial lactacidosis (Fig. 1). The relative flow rate ($V_{eq}$) of RBC’s was significantly increased starting at a dose of $10^{-5}$ mol/liter HWA 285. The strongest effect compared with the untreated control was measured using doses of $3 \times 10^{-4}$ (75% improvement) or $10^{-3}$ mol/liter (96%) HWA 285.

It was the aim of a further study to show that HWA 285 also has an ex vivo effect on hemorheology. In this connection, three experimental groups have been investigated and compared: (a) healthy, untreated control rats; (b) adjuvant arthritic rats (AAR); and (c) AAR that have been orally administered HWA 285 for 21 days at different doses (AAR + HWA 285). Different hematological and hemorheological parameters were measured and compared.

All hematological parameters showed significant differences between control and AAR (Table 1). A strong reduction in hematocrit and erythrocyte count combined with a decrease in mean cell volume (MCV) could be measured for AAR as compared with the untreated controls. Furthermore, the number of platelets was doubled. The strong increase in the leucocyte count in AAR reflects their involvement in inflammation. Various hematological parameters are altered by the administration of HWA 285: The number of platelets decreased and in some cases the erythrocyte count increased. No indirect rheological effect was observed on normalization of erythrocytic MCV. The leucocyte count decreased only with the highest dose of 50 mg/kg. No changes in osmolarity or electrolytes (Na⁺, K⁺, and Ca²⁺) were noted. On the contrary, an increase in the lactate values from AAR (2.73 ± 1.43 mmol/liter) as compared with the control rats (1.80 ± 0.85 mmol/liter) was measured. HWA 285 (1 and 10 mg/kg) slightly decreased the lactate values (2.67 ± 1.19 and 2.43 ± 1.03 mmol/liter). Results connected with higher doses of HWA 285 are missing for technical reasons.

The levels of total plasma protein, mainly fibrinogen, albumin, α₂-globulin, and γ-globulin are significantly changed in AAR compared to the control (Table 2). The strong rise in γ-globulins is caused by the inflammatory process. Values are not significantly influenced by the administration of propentofylline.

The increase in the plasma protein fractions fibrinogen and α₂-globulin accompanied by the decrease in albumin caused a significant rise in plasma viscosity for AAR (Table 3). Essentially, the drug did not affect plasma viscosity, with the exception of a significant increase in the plasma protein fractions fibrinogen and α₂-globulin.
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Fig. 1. Relative flow rate \( \left( V_{rel} = \frac{V_{susp}}{V_{buffer}} \right) \) of RBC's (dog) in the Nuclepore® filtration test (pore diameter 3 μm). Blood was incubated (in vitro) with different doses of propentofylline and afterward subjected to lactacidotic conditions (pH 6.9). \( V_{rel} \) was significantly improved starting at a dose of \( 10^{-6} \) mol/liter compared to the control \((n = 8, \text{each filtered five times})\).

TABLE 2. Plasma Proteins of Control Rats AAR \((n = 30)\) and AAR + Propentofylline \((n = 10)\) at the 21st Day of the Disease

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg p.o.)</th>
<th>Total protein (g/liter)</th>
<th>Fibrinogen (mg/100 ml)</th>
<th>Albumin (%)</th>
<th>( \alpha_2 )-Globulin (%)</th>
<th>( \gamma )-Globulin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>5.51*</td>
<td>81.8*</td>
<td>47.6*</td>
<td>4.9*</td>
<td>8.6*</td>
</tr>
<tr>
<td>AAR</td>
<td>—</td>
<td>6.16</td>
<td>581.2</td>
<td>16.9</td>
<td>14.3</td>
<td>16.7</td>
</tr>
<tr>
<td>AAR + Propentofylline</td>
<td>1.0</td>
<td>6.53</td>
<td>565.4</td>
<td>16.7</td>
<td>16.8</td>
<td>16.4</td>
</tr>
<tr>
<td></td>
<td>5.01</td>
<td>6.36</td>
<td>662.7</td>
<td>17.2</td>
<td>15.6</td>
<td>17.5</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>6.03</td>
<td>581.8</td>
<td>17.2</td>
<td>13.9</td>
<td>16.7</td>
</tr>
<tr>
<td></td>
<td>15.85</td>
<td>6.22</td>
<td>736.0</td>
<td>16.4</td>
<td>13.7</td>
<td>17.7</td>
</tr>
<tr>
<td></td>
<td>31.5</td>
<td>6.00</td>
<td>598.3</td>
<td>17.5</td>
<td>11.6</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>50.0</td>
<td>6.20</td>
<td>530.6</td>
<td>18.8</td>
<td>12.8</td>
<td>18.2</td>
</tr>
</tbody>
</table>

*Significant compared to AAR \((P < 0.05)\).

TABLE 3. Mean Values and Standard Deviation (SD) of Plasma Viscosity \((\eta_{pl}, \text{mPa s})\) for Control, AAR, and AAR Treated With Propentofylline (AAR + Propentofylline)

<table>
<thead>
<tr>
<th>( \eta_{pl} )</th>
<th>Control</th>
<th>AAR</th>
<th>AAR + propentofylline (mg/kg per os for 21 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \bar{x} )</td>
<td>1.116</td>
<td>1.450</td>
<td>1.476 1.463 1.408 1.430 1.336 1.477</td>
</tr>
<tr>
<td>SD</td>
<td>0.020</td>
<td>0.040</td>
<td>0.027 0.118 0.067 0.040 0.075 0.059</td>
</tr>
<tr>
<td>( p )</td>
<td>a</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

*aSignificant \((P < 0.05)\).

bNot significant, \( n = 10 \).
TABLE 4. Mean Extent of RBC Aggregation (MEA) for Control, AAR, and AAR Treated With Propentofylline (AAR + Propentofylline)

<table>
<thead>
<tr>
<th>AAR + propentofylline (mg/kg per os for 21 days)</th>
<th>MEA</th>
<th>Control</th>
<th>AAR</th>
<th>1</th>
<th>5.01</th>
<th>10.0</th>
<th>15.85</th>
<th>31.5</th>
<th>50.0</th>
</tr>
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<tbody>
<tr>
<td>x</td>
<td>5.30</td>
<td>23.22</td>
<td>21.12</td>
<td>23.47</td>
<td>22.98</td>
<td>20.82</td>
<td>21.52</td>
<td>22.37</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>1.42</td>
<td>3.36</td>
<td>4.83</td>
<td>6.15</td>
<td>6.59</td>
<td>8.73</td>
<td>3.72</td>
<td>4.94</td>
<td></td>
</tr>
<tr>
<td>P a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant (P < 0.05).
*Not significant, n = 10.

TABLE 5. Mean Passage Time (tM in ms) of Single RBC’s in the Single Erythrocyte Rigidometer (SER) With a Pore Diameter of 3.5 ± 0.1 μm for Control Rats, AAR, and AAR Treated for 21 Days with Propentofylline (The Drug was Administered p.o. for 21 days)

<table>
<thead>
<tr>
<th>tM</th>
<th>Control</th>
<th>AAR</th>
<th>5.01</th>
<th>15.85</th>
<th>31.5</th>
<th>50.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>x</td>
<td>30.80</td>
<td>33.85</td>
<td>33.74</td>
<td>33.53</td>
<td>15.64</td>
<td>29.43</td>
</tr>
<tr>
<td>SD</td>
<td>1.54</td>
<td>3.33</td>
<td>6.21</td>
<td>4.83</td>
<td>1.51</td>
<td>2.01</td>
</tr>
<tr>
<td>% improv.</td>
<td>12</td>
<td></td>
<td>46</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P a</td>
<td></td>
<td></td>
<td>b</td>
<td>b</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>P b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Significant (P < 0.05).
*Not significant, n = 10.

reduction in high plasma viscosity after oral administration of 31.5 mg/kg of HWA 285 for 21 days to AAR.

The medium extent of MEA was significantly increased in AAR compared to the control (Table 4). At the doses administered, HWA 285 did not affect MEA. Diminished RBC fluidity in AAR was shown by three different methods. Reduced erythrocyte deformability in AAR was, however, significantly improved by propentofylline in all three methods.

Relative flow rate ($V_{rel}$) of RBC suspensions through Nuclepore filters was significantly increased starting at an oral dose of 15.85 mg/kg (Fig. 2). The initial slope of the flow curve in the Filtrometer was significantly improved even at the lower dose of 10 mg/kg (Fig. 3). Furthermore, even single RBC deformability was significantly increased in AAR’s compared with untreated controls starting at an oral dose of 31.5 mg/kg for 21 days (Table 5). In all three test systems, a lower effect on deformability was observed at the highest dose of 50 mg/kg. Oral administration of 15.85 and 31.5 mg/kg of HWA 285 for 21 days achieved the maximum improvement in RBC deformability in AAR’s.

DISCUSSION

The hemorheological effects of the drug HWA 285 were investigated in different models that are supposed to simulate the pathophysiological conditions of disturbed blood perfusion. A protective effect of HWA 285 against RBC rigidification artificially induced by lactacidosis could be demonstrated. The relative flow rate of RBC in the Nuclepore filtration test increased dose-dependently starting at a dose of $10^{-5}$ mol/liter. Furthermore, HWA 285 improved the deformability of rigid RBC’s from AAR’s in all three methods. No effect of the drug on plasma viscosity and medium extent of RBC aggregation was measured.

RBC fluidity is affected by several factors; the metabolic state (i.e., the pH value and the lactate concentrations) plays an important role in microcirculatory perfusion. Dintenfass
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Fig. 2. Improved (\%) relative flow rate ($V_{rel} = V_{sus}/V_{buffer}$) of RBC's (rat) in the Nuclepore® filtration test (pore diameter 3 \textmu m) after oral administration of propentofylline for 21 days at different doses to AAR compared to untreated controls ($n = 10$, each filtered five times). Significant improvement of $V_{rel}$ starting at a dose of 15.85 mg/kg.

Fig. 3. Improvement (\%) of initial slope (init. slope) of the flow curve of RBC's in the Filtrometer MF 4 through Nuclepore® filters (pore diameter 3 \textmu m). Propentofylline was administered orally for 21 days at different doses to AAR and compared with untreated controls ($n = 10$, each tested four times). Significant improvement of initial slope starting at a dose of 10 mg/kg.

[1971] has observed a pH decrease during the inversion of the "Fahreus-Lindquist effect," that is, the sudden stasis in narrow capillaries, whereby changes in RBC deformability will occur. The rigidification of the RBC's in the in vitro and ex vivo tests is dependent on the concentration of lactic acid in the blood. HWA 285 improved RBC deformability in vitro as well as ex vivo as revealed by various hemorheological tests. Not only the complex filtration rate through Nuclepore filters was increased, but also single erythrocyte passage time in the SER, which means that the drug has a direct hemorheological effect even on single RBC's. The significant
decrease in the thrombocyte count (Table 1) indicates an effect on platelet function that still has to be explored. These studies are of interest as they help to explain the blood-flow-improving effect of HWA 285 in critically hypoperfused vascular beds either in peripheral or in cerebral blood flow disturbances.

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REFERENCES


