Cilostazol and blood viscoelasticity in homozygous sickle cell disease

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Homozygous sickle cell anaemia is an hereditary disease resulting from the single point mutation within the gene that encodes the haemoglobin β-globin chain. Abnormal β-globin chains are produced when a substitution occurs in the second nucleotide of the sixth codon of this gene, resulting in the changing of a glutamine molecule with a valine molecule. When the blood becomes deoxygenated, the valine can form non-covalent (hydrophobic) interactions with neighbouring haemoglobin chains, resulting in polymerisation of the HbS. This is the molecular hallmark of sickle cell disease.

Groups of insoluble polymers may aggregate causing distortion of the red blood cells into a sickle shape.[1] These red blood cells assume an abnormal, rigid and crescent or sickle shape with decreased flexibility. Polymerised haemoglobin is injurious to the red cell membrane, resulting in cellular dehydration, oxidative damage and increased adherence to endothelial cells.[2]

A cardinal sign of sickle cell disease is the occurrence of vaso-occlusive crisis where sickled red blood cells block small blood vessels, thereby depriving tissues of oxygen and leading to organ damage, especially in the brain, lung, kidney or spleen.[3]

Deformability describes the ability of the erythrocyte to change its shape in response to a distorting force. Red cell deformability is influenced by three distinct cellular components: cell shape geometry, viscosity of the cell cytoplasm, and stability of the membrane.[4] If any of these parameters are altered, the deformability of the erythrocyte is impaired and the ability of red blood cells to pass through small arterioles or capillaries is compromised. This ultimately prevents oxygen transport to the tissues and may result in hypoxia.

Blood exhibits pronounced elasticity and viscosity, and these viscoelastic properties affect its pulsatile flow.[5] Viscosity refers to an assessment of the rate of energy dissipation due to red blood cell deformation and sliding, while elasticity is an assessment of the elastic storage of energy, primarily in the kinetic deformability of the red cells.[6] Cells with less elastic storage energy will require less energy for deformation and are therefore more deformable; the higher the elasticity of the membrane, the more rigid it is and therefore the less deformable. The relaxation time relates to the speed at which red cells can change their shape and orientation in response to changes in the circulation. An increase in relaxation time indicates a decrease in the speed at which cells can enter the microcirculation.[7]

Cilostazol – 6-(4-[l-cyclohexyl-1H-tetrazol-5-yl] butoxy)-3, 4-dihydro-2(1H)-quinolinone – is a potent antithrombotic drug with vasodilatory properties. Currently, it is used extensively in the treatment of intermittent claudication, a form of peripheral arterial disease.[8] The general physiological effects of cilostazol include increased cardiac contractility and vascular smooth muscle cell relaxation, decreased platelet aggregation and vascular smooth muscle proliferation.[9] It selectively inhibits phosphodiesterase isoenzyme III (PDE3), the enzyme responsible for inactivation of the second messenger cyclic adenosine monophosphate (cAMP).

Intracellular concentrations of cAMP are regulated by adenylate cyclase and PDE3 activity,[10] and cAMP controls the intracellular calcium concentration by its modulation of ATP concentration.[11] Inhibition of PDE3 will result in increased cAMP concentration, cause an increase in ATPase activity and thus a decrease in intracellular calcium concentration.[12] Therefore it is expected that cilostazol will cause a decrease in the intracellular calcium concentration, resulting to an increase in the deformability of sickle red blood cells. Recently, the presence of PDE3, which controls the intracellular concentration of cAMP, has been demonstrated in erythrocytes.[13]

The present small study aims to investigate the effect of cilostazol on the viscoelasticity of blood in homozygous sickle cell disease.

One 50-mg tablet of Pletoz (cilostazol; Cipla, India) was dissolved in a 100 mL solution of dimethyl sulphoxide (DMSO) and water (1:9 ratio) to form a stock solution of 0.5 mg/mL. Aliquots of 10, 20, 40 and 80 μL were pipetted from the stock solution into Eppendorf tubes with 1 mL blood to constitute representative doses of 50, 100, 150 and 200 mg, respectively. The control tube had no added drug. The mixtures were allowed to incubate for 20 minutes at room temperature (25°C) before measurements were taken.

Ten patients with homozygous sickle cell disease (HbSS) were recruited from the sickle cell clinic of the University of the West Indies at Mona. Age ranged from 19 to 23 years, with a haematocrit value of 27.26±1.9%. An equal number of age-matched subjects with normal haemoglobin (HbAA) and a haematocrit value of 41.6±1.6% recruited from the Department of Basic Medical Sciences, University of the West Indies, acted as controls.

Informed consent was obtained from each subject and control before recruitment into the study. The study was performed according to the guidelines of the Helsinki Declaration and was approved by the Faculty of Medical Sciences/University of the West Indies Ethics Committee. Patients in sickle crisis were not included in this study.

Venous blood (10 mL) was drawn from the antecubital vein into Vacutainer tubes containing K+ EDTA (1.5 mg/mL) and stored at room temperature (25°C) until measurements were taken. Haematocrit values were measured using a Coulter Counter (Coulter, Miami, Florida). The value of the haematocrit was fed into the BioProfiler (Vilastic Scientific) before viscoelasticity and relaxation times were measured.

Blood viscoelastic properties were analysed using the BioProfiler, which is capable of giving single measurements of viscosity, elasticity and relaxation time of whole blood at shear rates of 2.51 sec^{-1}, 12.6 sec^{-1} and 62.8 sec^{-1}, and strain of 0.2, 1 and 5, respectively.[14] Measurements were taken at a frequency of 2 Hz and a shear rate of 62.8 sec^{-1}, and strain of 5 at 37°C at both native haematocrit and a projected haematocrit of 45%. All measurements were taken within

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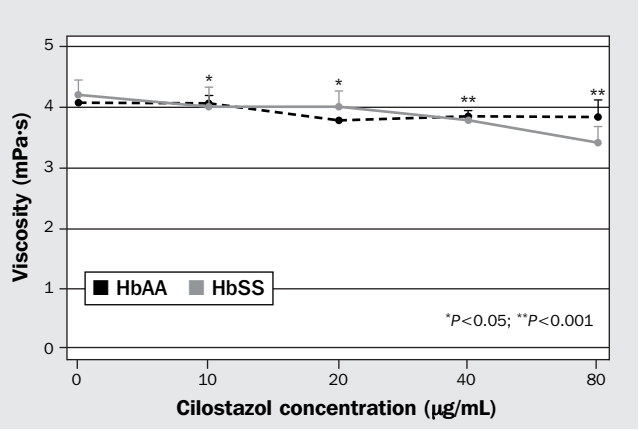

Fig. 1. The effects of different concentrations of cilostazol (mean±SEM) on viscosity in subjects (HbSS) and controls (HbAA) at a projected (45%) haematocrit.

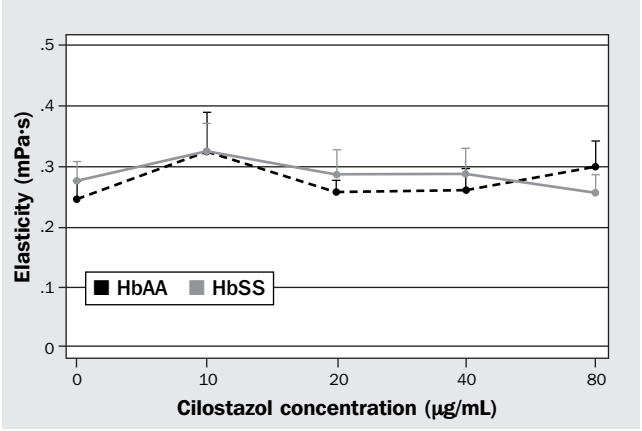

Fig. 2. The effects of different concentrations of cilostazol (mean±SEM) on elasticity in subjects (HbSS) and controls (HbAA) at a projected (45%) haematocrit

60 min of sample collection. The BioProfiler is able to give readings at a standardised or projected haematocrit of 45%.

Data were analysed using Stata version 10.1 for analysis of variance (ANOVA) and paired Student's *t*-test. $P<0.05$ was considered significant. The values for viscosity, elasticity and relaxation time at native haematocrit are shown in Table 1, while their corresponding values at a projected haematocrit of 45% are shown graphically (Figs. 1, 2 and 3).

Viscosity among the HbSS subjects was significantly ($P<0.001$) lower than that of the HbAA controls at native haematocrit, due to the lower haematocrit levels in the subjects (Table 1). At the projected haematocrit, the viscosity of the HbSS subjects increased compared the HbAA controls as a result of reduced cell deformability in the former, although this was not statistically significant. The viscosity values in both groups decreased with increasing concentrations of cilostazol, reaching statistical significance ($P<0.001$) in the HbSS subjects but not in the HbAA controls (Fig. 1).

Elasticity among the HbSS subjects was significantly ($P<0.001$) lower than in the HbAA controls at native haematocrit, due to the lower haematocrit levels in the subjects (Table 1). At the projected haematocrit, there was a marginal increase at 10 μg/mL cilostazol, followed by decreases with increasing cilostazol concentration in the HbSS subjects. However, these changes did not achieve statistical significance. In the HbAA controls, elasticity increased with increasing concentrations of cilostazol, reaching a peak at 20 μg/mL before declining marginally. Similarly, these changes did not achieve statistical significance (Fig. 2).

The relaxation time in HbSS subjects was lower than in the HbAA controls at native haematocrit; however, this did not achieve statistical significance (Table 1). At the projected haematocrit, there was a significant increase in the relaxation time at a cilostazol concentration of 10 μg/mL. This was followed by decreases with increasing concentration in the HbSS subjects. In the HbAA controls, relaxation time increased with increasing cilostazol concentration, reaching a peak at 20 μg/mL, before declining marginally; however, this did not achieve statistical significance (Fig. 3).

Cilostazol caused a significant dose-dependent decrease in blood viscosity in the HbSS subjects, while the decrease observed in the control (HbAA) subjects did not achieve statistical significance. The ability of cilostazol to lower blood viscosity both in subjects and control is in agreement with a previous report that showed a decrease in whole blood viscosity with cilostazol but not with pentoxifylline administration.[15]

Elasticity both in HbSS subjects and HbAA controls increased marginally and then declined with increasing cilostazol concentration; however, these changes also did not achieve statistical significance. The effect of cilostazol on elasticity did not seem to affect viscosity, as there were initial marginal increases in elasticity, with a peak at 10 μg/mL for the subjects and 20 μg/mL for the controls before declining marginally. The cilostazol concentration at which elasticity peaked both in subjects and controls corresponded to the point at which viscosity dipped.

Table 1. Viscosity, elasticity and relaxation time (mean±SEM) at different cilostazol concentrations for subjects (HbSS) and controls (HbAA) at native haematocrit.

Cilostazol (μg/mL)	Viscosity @62.8 sec^{-1} (mPa·s)		Elasticity @62.8 sec^{-1} (mPa·s)		Relaxation time (sec)	
	HbSS ($n=10$)	HbAA ($n=10$)	HbSS ($n=10$)	HbAA ($n=10$)	HbSS ($n=10$)	HbAA ($n=10$)
0	2.85 (0.17)*	3.85 (0.26)	0.115 (0.02)	0.207 (0.03)	0.0031 (0.0005)	0.0042 (0.0005)
10	2.78 (0.18)†	3.85 (0.25)	0.122 (0.02)	0.273 (0.05)	0.0035 (0.0005)	0.0057 (0.001)
20	2.77 (0.17)‡	3.60 (0.30)	0.105 (0.01)	0.303 (0.08)	0.0030 (0.0003)	0.0087 (0.004)
40	2.6 (0.15)*	3.65 (0.23)	0.111 (0.02)	0.218 (0.03)	0.0032 (0.0005)	0.0048 (0.0006)
80	2.57 (0.15)*	3.56 (0.26)	0.101 (0.03)	0.255 (0.04)	0.0031 (0.0005)	0.0055 (0.0006)

*$P<0.001$; †$P<0.05$; ‡$P<0.01$

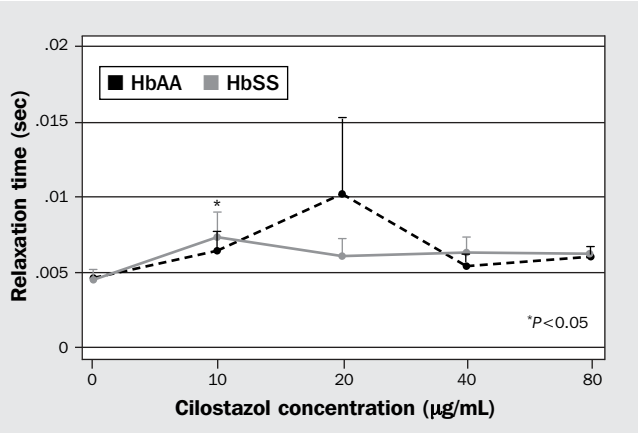

Fig. 3. The effects of different concentrations of cilostazol (mean±SEM) on relaxation time in subjects (HbSS) and controls (HbAA) at a projected (45%) haematocrit.

The increase in elasticity can be explained by the role of cAMP in causing an initial increase and then a decrease in membrane deformability. At concentrations between 10^{-11} mmol/L and 10^{-10} mmol/L, cAMP caused erythrocyte membrane to be 'loosened or relaxed' due to interaction with its receptors, while at concentrations between 10^{-10} mmol/L and 10^{-8} mmol/L it caused the erythrocyte membrane to be 'stiffened' due to activity of the protein kinase system and phosphorylation of the membrane proteins.[16]

If the increase in elasticity observed in the present study were to have had any significant effect on whole blood viscoelasticity, it should have caused an increase in blood viscosity. In a previous study,[17] it was observed that a significant increase in erythrocyte elasticity with increasing doses of dibenzyl trisulphide (DTS) resulted in an increase in blood viscosity.[18]

Relaxation time for the HbSS subjects increased significantly at 10 μg/mL cilostazol, but did not achieve significant increase in the HbAA controls. A similar trend was also observed for elasticity. It is noteworthy that a significant drop in blood viscosity was observed at this concentration in the HbSS subjects.

An increase in relaxation time indicates a decrease in the speed at which cells can enter the microcirculation.[7] An elevated or increased relaxation time suggests increased erythrocyte disaggregation, which could be extrapolated to mean a reduction in erythrocyte aggregation. Increased erythrocyte aggregation leads to an increase in blood viscosity.[19] Conversely, a decrease in erythrocyte aggregation, as suggested in this study, will predispose to a decrease in blood viscosity. The significant increase in relaxation time observed at a cilostazol concentration of 10 μg/mL in the HbSS subjects corresponded with a significant decrease in blood viscosity at the same concentration.

Platelet aggregation[20] and erythrocyte aggregation[21] involve the action of large molecular weight proteins such as fibrinogen. Cilostazol has been reported to inhibit platelet aggregation,[22] and it is possible that it might inhibit erythrocyte aggregation, thereby causing increased erythrocyte disaggregation. The results from the present study suggest that cilostazol inhibits erythrocyte aggregation by virtue of the increased relaxation time observed. Therefore, this could be the mechanism through which it decreases whole blood viscosity, thereby improving blood flow.

In conclusion, the results of the present study show that cilostazol reduces whole blood viscosity, possibly as a result of its effect in increasing relaxation time. This action complements the role of cilostazol in platelet disaggregation and could be beneficial in the improvement of blood flow in patients with intermittent claudication.

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Urease production as a marker of virulence in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa remains an intractable problem as a cause of respiratory infection in cystic fibrosis (CF) patients. While respiratory infection with *P. aeruginosa* in the majority of non-CF patients represents an acute, potentially fatal illness with risk of systemic dissemination, in CF patients it represents a chronic colonisation syndrome in which much of the local tissue damage is due to host response to the bacteria, rather than metabolic products of the bacteria themselves.

In the CF lung, *P. aeruginosa* grows in a biofilm state under anaerobic conditions.[1] After extended infection in the lung, CF isolates of *P. aeruginosa* are often hypermutable,[2] and mutation of regulator genes may occur over an extended period of colonisation, leading to a relatively dormant, slow growing phenotype. Strains show a gradual but marked change over time towards mucoid, non-motile and antibiotic resistant phenotypes.[3]

In recent decades, multidrug-resistant clonal strains of this organism have been described infecting CF patients on several continents.[4–10] At least one clonal strain (Australian epidemic strain 3; AES3) has been shown to result in adverse clinical outcomes for patients.[10] The mechanisms by which such clonal strains establish infection in the CF lung, and sometimes supplant other non-clonal isolates of *P. aeruginosa*, remain to be fully elucidated. Reliable mechanisms for the detection of these clonal strains in routine diagnostics are yet to be determined.

Ceftazidime resistance was noted as a marker for the Liverpool epidemic strain (LES) in 1996,[6] but not all LES strains are resistant to this antibiotic,[11] and non-clonal CF isolates may often express such resistance.[12]

P. aeruginosa isolates from CF respiratory infections also gradually cease to express virulence factors over time, leading to a less virulent phenotype, as demonstrated by immunoblot analysis of type III secretion system effector enzyme expression[13] and whole cell virulence in a *Dictyostelium discoideum* eukaryotic virulence model.[14] The progressive nature of whole cell virulence loss has also been demonstrated in a *D. discoideum* eukaryotic virulence model.[15]

Almost all non-CF isolates of *P. aeruginosa* express the enzyme urease. This enzyme acts to catalyse the hydrolysis of urea, producing ammonia and carbamic acid, which is in turn hydrolysed to form bicarbonate. This enzyme is produced by numerous pathogenic bacteria and fungi, and has recently been the focus of interest as a major virulence factor in some respiratory pathogens.[16] In *Cryptococcus neoformans*, pH changes due to ammonia derived from urease activity have a role in immune evasion.[16] Ammonia is toxic to host cells, promoting dissemination from the lung to the bloodstream and to other organs.[16] Urease production in *Mycobacterium bovis* assists immune evasion by decreasing both localisation in the lysosome and cell surface expression of major histocompatibility complex class II.[16] In *P. aeruginosa*, urease plays a role in pH homeostasis within biofilm growth, and *ureB*-negative mutants show poor biofilm formation.[17] Expression of urease in *P. aeruginosa* is controlled by *rpoN*.[18]

Progressive loss of *rpoN* expression of dependent surface factors in *P. aeruginosa* has been shown to occur during the course of colonisation of the CF lung.[19] Down-regulation of *P. aeruginosa* Sigma-factor 54 (σ^{54}), coded by *rpoN*, has been described in the Danish CF clonal strain DK2. It has been postulated that a combination of mutations in *rpoN*, *mucA* and *lasR*, possibly combined with mutations conferring resistance to antibiotics, have resulted in the success of this clonal strain in colonising and disseminating within the Danish CF population.[3] Such mutants are slower growing and have defects in flagella and type IV pili, resulting in a non-motile phenotype.

Knockout mutation in *rpoN* will have a significant impact on quorum sensing[20] and virulence in *P. aeruginosa*, as well as halting urease and glutamate synthase expression.[18] Additionally, pilin[21] and flagella[18] genes will not be produced, leading to a non-motile phenotype. Expression of *lasR* and *lasI* is controlled by *rpoN*, and *rpoN*-negative mutants show increased production of these genes at low cell densities, but these effects are reversed at high cell densities. Expression of *lasB* (coding for elastase) *hcnA* (cyanide synthase) and *rhlA* (precursor of rhamnolipid) is derepressed in such mutants.[20] Loss of *rpoN* function has also been shown to confer the capacity for growth under anaerobic conditions, as found in the CF lung.[1]

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