Relating the blood-thinning effect of pentoxifylline to the reduction in the elastic modulus of human red blood cells: An *in vivo* study

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Abstract

The blood thinning properties of pentoxifylline have been attributed to its ability to increase the deformability of red blood cells and improve their rheological properties. To interpret and substantiate these observations a novel approach is taken by measuring the stiffness of individual red blood cells from healthy humans before and after subscription to pentoxifylline for nine days. Atomic force microscopy nanoindentation experiments reveal that the elastic modulus of red blood cells decreased by 30%-40%, after pentoxifylline subscription. This decrease in elastic modulus is related to the ability of pentoxifylline to increase the production of ATP and decrease Ca2+ concentrations in red blood cells. The present experiments are the first to prove that a widely used blood thinning medication, pentoxifylline, indeed reduces the elastic modulus of healthy human red blood cells, paving the way to use indentation in medicine. It is also encouraging that this study was performed on healthy volunteers.

1. Introduction

Pentoxifylline (PTX) (3,7-Dihydro-3,7-dimethyl-1-(5-oxohexyl)-1H-purine-2,6-dione) is a xanthine derivative drug, which inhibits erythrocyte phosphodiesterase, resulting in an increase in erythrocyte cAMP activity. PTX is routinely prescribed to patients for the prevention of stroke episodes, as it enhances the blood flow velocity through the small blood vessels[1], such as brain capillaries. With its hemorheological

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activity, PTX is used to treat various diseases, such as cerebrovascular insufficiency, senile organic brain syndrome, transient ischemic attacks, ocular and otological circulatory disorders, as well as peripheral angiopathies with intermittent claudication[2].

In vivo and *vitro* studies have concluded that PTX increases the red blood cell (RBC) deformability[3-4], K. Słoczyńsk, M. Kózka, E. Pękala, A. Marchewka and H. Marona, In vitro effect of pentoxifylline and lisofylline on deformability and aggregation of red blood cells from healthy subjects and patients with chronic venous disease Acta Biochimica Polonica 60, 129–135 **(2013)**, which allows for an increase in the blood flow velocity, and a decrease in local hyperviscosity and hyper-aggregability of RBCs[1-2,5]. As a result the oxygenation of tissues is improved and, rouleau leading in clots are avoided[1-5].

More recently, therefore, in addition to prescribing PTX to patients with cerebrovascular and peripheral arterial disease, it is also given to malaria patients, since plasmodium penetration reduces significantly the RBC membrane plasticity and deformability, making blood flow difficult. *In vitro* use of PTX on malaria infected RBCs showed an increase in the blood flow velocity through pipettes, suggesting an increase in the RBC deformability[6]. In this context it should be emphasized that PTX is also considered for the treatment Alzheimer's Ekert JO, Gould RL, Reynolds G, Howard RJ. TNF alpha inhibitors in Alzheimer's disease: A systematic review. International journal of geriatric psychiatry. 2018 May;33(5):688-94), which has been shown to increase RBC rigidity Bester, J., Buys, A.V., Lipinski, B., Kell, D.B., Pretorius, E.: High ferritin levels have major effects on the morphology of erythrocytes in Alzheimer's disease. Front. Aging Neurosci. 5, 88 (2013).

Most of the aforementioned studies are *in vitro* and incubate RBCs with PTX in order to assess its effects on the RBC rheology or deformability Ehrly AM: The effect of pentoxifylline on the flow properties of hyperosmolar blood. ICRS Medical Science 3:465, 1975; Sowemimo-Coker SO, Turner P: The effect of pentoxifylline on filterability of normal red blood cells and their adhesiveness to cultured endothelial cells. Eur J Clin Pharmacol 29:55-59, 1985, K. Słoczyńsk, M. Kózka, E. Pękala, A. Marchewka and H. Marona, In vitro effect of pentoxifylline and lisofylline on deformability and aggregation of red blood cells from healthy subjects and patients with chronic venous disease Acta Biochimica Polonica 60, 129–135 **(2013)**. Deformability is usually measured by examining the RBC filterability through membranes of a fixed pore size Sowemimo-Coker SO, Turner P: The effect of pentoxifylline on filterability of normal red blood cells and their adhesiveness to cultured endothelial cells. Eur J Clin Pharmacol 29:55-59, 1985, or by measuring the deformability index and elongation through a laser-assisted optical rotational cell analyser K. Słoczyńsk, M. Kózka, E. Pękala, A. Marchewka and H. Marona, In vitro effect of pentoxifylline and lisofylline on deformability and aggregation of red blood cells from healthy subjects and patients with chronic venous disease Acta Biochimica Polonica 60, 129–135 **(2013)**.

Such studies, although efficient in establishing that PTX affects the mechanical properties of RBCs, are more of a qualitative nature as deformability and elongation are not material parameters used to define elastic behavior. This 'increase in deformability' of RBCs that is used in medical terminology can be translated to a 'decrease in the elastic modulus', or 'increase in elasticity' in rigorous scientific terminology. The elastic modulus is one of the most explored mechanical properties that define the behavior of materials and well-established techniques have been development for its measurement, such as atomic force microscopy (AFM) indentation that will be used herein.

The advantage of AFM is that in addition to being more accurate, it images the sample surface prior to indenting, and the tests can be performed in a liquid environment allowing to maintain physiological conditions. Therefore, numerous studies employ AFM to measure the elastic modulus of RBCs Ref[10], Atomic force microscopy imaging and mechanical properties measurement of red blood cells and aggressive cancer cells November 2012 Vol.55 No.11: 968–973, Ida Dulinska, Marta Targosz, Wojciech Strojny, Maygorzata Lekka, Pawey Czuba, Walentyna Balwierz, Marek Szymonski J. Biochem. Biophys. Methods 66 (2006) 1 – 11, J L Maciaszek, B Andemariam, G Lykotrafitis, Microelasticity of

red blood cells in sickle cell disease, J. Strain Analysis Vol. 46, 368-379, 2011 and examine how it's value can be affected by various diseases Ida Dulinska, Marta Targosz, Wojciech Strojny, Maygorzata Lekka, Pawey Czuba, Walentyna Balwierz, Marek Szymonski J. Biochem. Biophys. Methods 66 (2006) 1 – 11, J L Maciaszek, B Andemariam, G Lykotrafitis, Microelasticity of red blood cells in sickle cell disease, J. Strain Analysis Vol. 46, 368-379, 2011. In order to perform AFM in a liquid cell the RBCs must be fixed onto the substrate. Therefore, all studies that use AFM to measure the elasticity of RBCs first treat the substrate with poly-l-lysine in order to increase cell adherence, and fixation of the RBCs is performed by treating them with glutaraldehyde. Then PBS buffer solution is added to avoid dehydration and indentation is performed to obtain the elastic modulus through the Hertzian method. The drawbacks of this procedure are that glutaraldehyde kills the RBCs, and the Hertzian method cannot account for viscoelastic effects. In the present study both of these drawbacks are overcome as (i) fresh blood samples are attached on glass slides by the smearing method, which allows direct attachment of RBCs, without the use of poly-l-lysine or glutaraldehyde, and (ii) the jump-rate method [7,8,9], which accounts for viscoelastic effects is used to measure the elastic modulus. Another novelty of the present study is that the PTX was administered *in vivo* to healthy human volunteers and their fresh blood were examined immediately after extraction, as opposed to incubating RBC with PTX for *in vitro* studies.

In concluding this introduction, it should be noted that since PTX can alter the elasticity of RBCs, it is implied that it can affect their microstructure. It was pointed out in an early study that PTX, as a derivative of methylxanthine, acts as an inhibitor to phosphodiesterase enzymes, which are found in mitochondria, and as a result can increase the interior membrane systems of the cell[14] (the membranes of mitochondria, smooth endoplasmic reticulum and Golgi complex), while at the same time it can restore plasma membrane deformation, which may occur either as a result of hypoxia or due to eventual energy deficiency. This was illustrated[14] by examining the mitochondria of neurons in the hippocampus and cerebral cortex of gerbils who were prescribed PTX after being made to suffer an ischemic condition. It was revealed that the PTX treated gerbils had an impressive hypertrophy of mitochondria in comparison with normal controls and untreated ischemic gerbils[14]. The effect of PTX on the structure of RBC mitochondria cannot be examined, as RBCs released into peripheral blood are mature and lack a nucleus and formed organelles. However, they still contain phosphodiesterase enzymes that can react with PTX, which can then affect the plasma elasticity. It is, therefore, important to illustrate that PTX does not only affect the mitochondrial structure of neurons but of other cells as well, since this could rationalize the increase in the RBC membrane flexibility. In the present study, hence, muscle mitochondria, which are more durable, and can be fixed in proper condition, were chosen to examine PTX on other cells. A Lewis rat (AgB-1) was therefore treated with 2 mg of PTX for two weeks, and electron microscopy was performed on its major gluteus muscle.

The present study is therefore able to capture through in vivo studies in humans and animals the structural and mechanical effects that PTX has on RBCs.

2. Experimental Methods

This study was approved and performed according to the IRB protocols of the Aristotle University of Thessaloniki, where the experiments took place.

2.1. In vivo human studies

In order to capture only the effect of PTX and not that of other physiological parameters, three healthy human subjects were used in this study that wished to start

taking PTX for athletic purposes; they were not on any other medications/substances, nor suffering from a medical condition. Prior to initiation of PTX, they gave a small blood sample (poke on thumb by a sterilized syringe), which was immediately smeared on a glass slide, so as to obtain a monolayer of live RBCs. The glass slide was then immediately placed in the atomic force microscope (AFM), where imaging and indentation were performed within ten minutes after blood extraction. After ten minutes the sample was disposed of and a fresh blood sample was obtained in order to obtain enough data for statistical purposes. For each subject 10 indentations were performed on 15 different RBCs, prior to PTX initiation. Then, the subjects began taking 400mg of PTX every 12 hours (800mg/day), for 9 days. On the 9th day the aforementioned AFM procedure was repeated so as to obtain the elastic modulus of RBCs with PTX effects.

2.2. Calculation of elastic modulus of RBCs

Imaging and indentation were performed using the Veeco Multimode AFM with 12 nm SiC tapping mode tips. After performing the AFM indentations the elastic modulus of the RBCs was deduced using the jump rate method[7-9], which has been developed for measuring accurately the elasticity of viscous materials such as soft polymers and cells. This protocol involves applying a step jump in the loading rate during loading. Only the elastic component of the material constitutive behavior responds to the step jump and, therefore, by subtracting the load-displacement response of the RBC just before and just after the rate jump, removes the contribution from the viscous deformation[8].

In an AFM the movement δ of the sample stage, and the photodiode signal D due to cantilever deflection, are recorded over time. During the imposed step jump in the loading rate, these two quantities undergo step changes in their time-rates of $\dot{\delta}$ and $\dot{D}$ respectively, which can be obtained from the recorded data. $\dot{\delta}$ and $\dot{D}$ have been shown to be related by[7]

$$\frac{\Delta\dot{\delta}}{\Delta\dot{D}} = A\left(1+\frac{\alpha}{E_r}\right), \tag{1}$$

where A is a sensitivity constant, i.e. the deflection per photo-diode signal of the AFM cantilever, and α is the ratio between the spring-stiffness of the AFM cantilever to the end-diameter of the AFM tip which is assumed to be flat-ended. The final parameter in Eq. (1) is E_r, which denotes the reduced elastic modulus of the tip-sample contact, and is given as

$$E_r = \left(\frac{1-\nu_{tip}^2}{E_{tip}} + \frac{1-\nu_{sample}^2}{E_{sample}}\right), \tag{2}$$

where ν_{tip}, E_{tip}, ν_{sample}, E_{sample}, are the Poisson's ratio and elastic modulus of the AFM tip and sample, respectively. Hence, for a particular cantilever-tip, the constants A and α can be determined by obtaining AFM indentation force-distance curves for two standard materials with known elastic moduli. Then the same tip can be used to indent a sample with unknown mechanical properties and Eqs (1) and (2) can be used to calculate E_{sample}, which in this case is the elastic modulus of the RBC (E_{RBC}).

This jump-rate method has been found to yield reliable results for the elastic modulus of numerous metal and polymer materials, and more recently for oral cancer cells[9]. It was therefore, employed in the present study, using a soft solid gel (E=0.6 MPa) and a Si wafer (E=150 GPa) as standards for determining A and α in Eq. (1). Before beginning the experiments, the AFM tip was flattened by indenting on the Si

wafer in order to achieve a flat-ended tip. After determining A and α from the Si and gel, a test sample of highly oriented pyrolytic graphite (provided by Veeco as a standard) was indented using the above rate-jump protocol and its elastic modulus was found, using Eqs (1) and (2), as 18 GPa. This value was in agreement with the value stated by Veeco, and hence verified that the jump rate method employed herein accurately measures the elastic modulus of materials. Once this verification was completed indentations on the RBCs began. Every time the tip was changed or a new day of experiments began A and α were determined from the gel and Si standards anew.

2.3. In vivo rat study

In order to examine the effect of PTX on mitochondria 2mg of PTX were prescribed for 15 days to a healthy Lewis rat (AgB-1) that was 3 months old. The rat was then anesthetized with iso 12 and injected transcardially with 1ml of Euthasol (390 mg/ml pentobarbital, 50 mg/ml phenytoin). Its major gluteus muscle was removed and initially placed in Sotelo fixing solution composed of: 1% paraformaldehyde, 2.5% glutaraldehyde in cacodylate buffer 0.1mol/L, and adjusted at a 7.35 pH. Then the specimens were fixed by immersion in 1% osmium tetroxide for 30 minutes at room temperature and dehydrated in graded alcohol solutions and propylene oxide. Thin sections were cut in a Reichert ultratome, contrasted with uranyl acetate and lead citrate, and studied in a Zeiss 9aS electron microscope.

3. Results

Figs 1a&b illustrate a blood sample smeared on a glass slide. It is seen that the RBCs are spread out in a monolayer. The smooth "donut" shape of the cells indicates they were alive. 25 minutes after extraction, the cell membrane began to rupture and asperities were detected as shown in Fig. 1c. Eventually, 50 minutes after extraction, a "star-like" shape was obtained as depicted in Fig. 1d. In order, hence, to ensure that all RBCs were at the same state once extracted, all measurements were performed on fresh blood samples within 10 minutes.

Fig. 2a presents a single RBC that was indented. The AFM indentation depth was kept below 50nm, which is well below 1/10 of the cell height (~800nm), and therefore the substrate elastic properties did not affect the measurements. Fig. 2b illustrates that the deflection signal of the cantilever underwent a spike as the tip penetrated into the cell and then retracted. This transition from indentation to retraction was taken to be the rate-jump point which was used in Eq. (1) to obtain E_r and subsequently in Eq. (2) to evaluation E_{RBC}.

Table 1 summarizes the elastic modulus before and after PTX subscription. It is seen that after the subjects had taken PTX for 9 days the elastic modulus of their RBCs decreased dramatically, by about 30-40%. On the other hand, the standard deviation corresponding to the repeated measurements from 15 randomly selected RBCs in each condition is small compared to the reduction in the average elastic modulus after PTX treatment. Therefore, although it is known that cells at different stages in their life cycles may behave differently mechanically[11], such effects are deemed small compared to the effects of the PTX. It is interesting to note that in all subjects considered the elastic modulus value was 3.5 times lower than its initial value after PTX subscription.

It should be noted that in prior studies[10] the elastic modulus of healthy RBCs was estimated using the Hertzian approach, which however, measures an apparent elastic modulus, that is derived from the rate by which the applied force increases with indentation depth. The Hertzian approach therefore provides the value of an apparent modulus is that contains the effects of viscosity and its value would change if the

loading rate were changed. In the aforementioned jump rate method[7-9] viscosity effects are decoupled from the measured elastic modulus, allowing thus the latter to be measured as an intrinsic property of the RBC independent of the loading conditions.

Fig. 3 depicts an electron micrograph of the major gluteus muscle of the Lewis rat which was subscribed PTX for 15 days. It is seen that the mitochondrial volume and cristae density increased, indicating that PTX can affect the membrane structure of mitochondria.

4. Discussion

The smearing method used to prepare the blood samples in this study, is widely employed by hematologists and is the most efficient technique to attach RBCs on a substrate. The drawback though of this method, is that it cannot allow for the AFM experiments to take place in a liquid environment, as the RBCs would detach. As mentioned in the introduction, in order to do AFM indentation in liquid it would have been necessary to process the blood in order to separate the RBCs and treat them with glutaraldehyde to achieve fixation, in addition to treating the substrate with poly-l-lysine[10]. This process, however, not only kills the cells, but poly-l-lysine and glutaraldehyde can affect the elastic modulus. Other techniques, such as optical or magnetic tweezers, which can measure the elastic modulus in liquid without the use of additional chemicals, provide very rough approximations for its value, so a comparison before and after PTX subscription could not be performed.

Since the experiments were not performed in a liquid environment, the RBCs were dehydrated. From a mechanics point of view it has been documented that all dehydrated biological tissues and cells are stiffer, and hence, have a significantly higher elastic modulus, from their respective hydrated state. It was, therefore, expected that the modulus values that would result from the present experiments would be in the MPa range, since that of hydrated RBCs is in kPas[10]. An earlier study used AFM to measure the elastic modulus RBCs that were fixated on the substrate using formaldehyde and then fully dehydrated and dried Bester, J., Buys, A.V., Lipinski, B., Kell, D.B., Pretorius, E.: High ferritin levels have major effects on the morphology of erythrocytes in Alzheimer's disease. Front. Aging Neurosci. 5, 88 (2013). The obtained elastic modulus was ~43,000 MPa, which is significantly higher than the values obtained prior PTX subscription in Table 1. Such a difference is expected since in our case the RBCs were not fully dehydrated and dried. Our results are therefore consistent with prior studies on dry RBCs.

The observed decrease of the elastic modulus after PTX treatment facilitates the deformability of RBCs, and can therefore explain *in vitro* observations, which have shown that RBCs in PTX can transit in microtubules at higher rates than normal cells[3-4]. Therefore, PTX can reduce the elastic modulus of RBCs which results in a micro-rheological profile improvement, as well as in an increase in blood transport capacity in subjects with cerebrovascular and peripheral arterial diseases.

PTX is known to have major effects on several membrane-associated activities in human blood cells, such as decreased superoxide generation activated by zymosan, formyl-methionyl-leucyl-phenylalanine, and concanavalin A, decreased uptake of adenosine, and increased clindamycin uptake in zymosan-stimulated polymorphonuclear neutrophils. As mentioned in the introduction administering PTX to gerbils who were made to suffer from an ischemic condition, by bilateral common carotid artery occlusion, resulted in an impressive hypertrophy of neuron mitochondria in comparison with normal controls and untreated ischemic gerbils[14]. Since then, a substantial body of evidence highlights the role of the mitochondrial inner membrane organizing system in protein biogenesis[15-16].

It can therefore be argued that the action of PTX on mitochondria is responsible for altering the elasticity of RBCs. In order for RBCs to be released into peripheral blood, they have to reach a mature state, at which they lack a nucleus and other formed organelles. At their premature state RBCs have the complete cellular structure, but with maturation the mitochondria enzymes that are responsible for oxidative phosphorylation and the respiratory function of the cell exist in a diffuse form in the cytoplasm and the plasma membrane. Without these enzymes the survival of RBCs would be impossible. Since the average life of an RBC in the periphery is 120 days, it follows that both the respiratory function of the cell and the production of energy by oxidative phosphorylation, are performed continuously. Hence, even though mitochondria are not present in peripheral RBCs PTX can still react with the diffuse phosphodiesterase enzymes, and affect plasma elasticity. In order, however, for this to occur, it must be shown that PTX can affect the mitochondria membrane structure of other cells, and not only neurons, as it has been shown thus far. Fig. 3 indeed illustrated that after prescribing 2mg of PTX to a Lewis rat(Igb1) for 15 days resulted in an increase in the number and the volume of mitochondria, as well as in the inner membranes in comparison with normal controls[17]. This is consistent with an in vivo study which indicated the ability of PTX to improve mitochondria function on patients with claudicating limb muscles[13], however, electron microscopy was not performed to illustrate if and how the mitochondria structure was affected.

It is therefore demonstrated that PTX can affect the mitochondria in other cells types as well. Muscle mitochondria, are very durable, and can be fixed in proper condition, and therefore were chosen for studying them under the electron microscope. Premature RBCs, which contain mitochondria, disintegrate shortly after extraction due to autolysis, and therefore it is very difficult to study them in the electron microscope.

The mechanism by which PTX can affect the deformability of RBCs is through the direct effect it has on mitochondria enzymes, which are responsible for providing mechanical stability, and hence dictate the plasticity and elasticity of membranes. In addition to altering the structure of mitochondria, it has been shown that PTX also increases the energy that they produce[5,18], resulting in (a) an increase of intracellular adenosine triphosphate (ATP) concentrations, (b) the decrease of intracellular Ca^{++} concentrations by activation of the Ca^{2+}- Mg^{2+} ATPase and calmodulin, (c) the increase in phosphorylation of the proteins in the erythrocytes membrane by facilitating Mg^{++} dependent phosphoprotein phosphatase and transglutaminase activity and (d) the increase of membrane phosphoprotein concentration. In mature RBCs, even though formed mitochondria do not exist, the change of monovalent and divalent ions is carried by the activation of two main pumps, which are found in the cytoplasm membrane of all cells; these pumps are the Na-K ATPase and the Ca^{2+}-Mg^{2+}ATPase. Hence, even with the lack of mitochondria, PTX can still increase ATP production and decrease Ca^{2+} concentrations in RBCs. This is in agreement with earlier studies which have also attributed the ability of PTX to alter the deformability of RBCs to its effect on ATP production Curr Med Res Opin. 1977;4(9):609-17. The effect of pentoxifylline on erythrocyte deformability and on phosphatide fatty acid distribution in the erythrocyte membrane. Schubotz R, Mühlfellner O.

The aforementioned increase in ATP production can be directly related to the decrease in the elastic modulus of the RBCs since it has been shown in theoretical and experimental studies, that a decrease in ATP results in a significant increase in the elastic modulus of cells[19]. Moreover, it has been shown that an increase in intracellular Ca^{2+} increases the modulus of RBCs[20], hence the decrease of Ca^{2+} concentrations due to PTX further contributes to a lower elastic modulus, and therefore higher flexibility of RBCs.

Our *in vivo* human and animal study provides further evidence for an ability of PTX to increase the elasticity of RBCs which is in agreement with earlier observations summarized in the introduction. The only study which is contradictory is that of Ballas and Cummings, Angiology, 1990, 41: 118-123, which did not observe a change in cell deformability after incubating RBCs in PTX. In their article Ballas and Cummings, Angiology, 1990, 41: 118-123, the authors suggested that the inconsistency observed might be due to a low PTX dosage used in the incubation, and yet they also stated that the measured deformability of RBCs taken from two patients with peripheral vascular disease, who had been taking PTX, did not change from the baseline, but they did not specify what the baseline was, and they gave no details on the experimental procedure. Furthermore, the deformability of the RBCs of the patients was not measured prior to the administering of PTX, so it cannot be known if PTX really reduced the RBC rigidity. Peripheral vascular disease causes a significant decrease in RBC flexibility Ambrus JL, Ambrus CM, Taheri SA, Gastpar H, Reddington MM, Taheri P, Kahn EA, Schattman GL, Dean LS, Moore RH. Red cell flexibility and platelet aggregation in patients with chronic obstructive vascular disease (COAD) and study of therapeutic approaches. Angiology. 1984 Jul;35(7):418-26. and hence the RBCs tested in Ballas and Cummings, Angiology, 1990, 41: 118-123 may have been more resilient to PTX effects. A recent study K. Słoczyńsk, M. Kózka, E. Pękala, A. Marchewka and H. Marona, In vitro effect of pentoxifylline and lisofylline on deformability and aggregation of red blood cells from healthy subjects and patients with chronic venous disease Acta Biochimica Polonica 60, 129–135 **(2013)**, whose results are consistent with ours, also noted that the inconsistency between their work and that of Ballas and Cummings, Angiology, 1990, 41: 118-123 may be due to the incubation temperature and experimental method used by them Ballas and Cummings, Angiology, 1990, 41: 118-123.

5. Conclusions

The present study has provided *in vivo* proof that PTX decreases the elastic modulus of red blood cells in healthy human subjects, which implies an increased flexibility of RBCs, allowing for the blood thinning benefits of PTX. After nine days of PTX subscription the RBC elastic modulus from healthy human subjects had decreased by 30-40%. In performing the experiments the cells were attached on the substrate using the smearing method, and therefore no external factors such as poly-l-lysine affected the measurements. Furthermore, it was shown that PTX increases the amount and volume of mitochondria in different types of cells, indicating that the increased plasticity and flexibility of RBC plasma membranes under the influence of PTX is related with the PTX enhancing effect on enzymes found in mitochondria that affect membranic stability and function. In RBCs, functions otherwise carried out in mitochondria, occur in the plasma membrane and the biochemical effects of PTX include an increase in ATP and decrease of Ca^{2+}, both of which have been shown to reduce the elastic modulus of cells.

In concluding, it should be noted that the present simplified technique of measuring the RBC modulus could be used in clinical studies in order to calculate the elasticity of patients's RBCs (before and after PTX subscription), allowing the determinination of the optimal PTX dosage that retains the modulus to the desired value. This will result in a minimization of the side effects of PTX. Another clinical application of the AFM indentation-RBC method, illustrated here, is the diagnosis of infections, as it has been shown that during infections the RBC flexibility decreases (elastic modulus increases)[21].

Acknowledgments

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Table 1 Average Elastic Modulus and Standard Deviation

	Before PTX		After PTX	
	E (MPa)	Standard Dev.	*E*(MPa)	Standard Dev.
Subject 1	390	10	110	6
Subject 2	910	9	260	5
Subject 3	840	7	240	5

Figure Legends

Fig. 1. 3D topography images of RBCs with AFM: (a) live cells, (b) live cells, c) early stage of 'dying' cells, d) later stage of 'dying' cells.

Fig. 2. (a) Single RBC chosen for indentation on membrane. (b) The change of DFL signal recorded on the RBC membrane during AFM indentation.The arrow indicates the rate-jump point across which Eq. (1) is applied to evaluate the elastic modulus of the cell.

Fig. 3. Electron micrograph indicating increased mitochondrial volume in the major gluteus muscle of a Lewis rat (Igb1) treated with 2 mg of PTX for two weeks. The top arrow indicates the mitochondria, while the lower one their internal membranes.

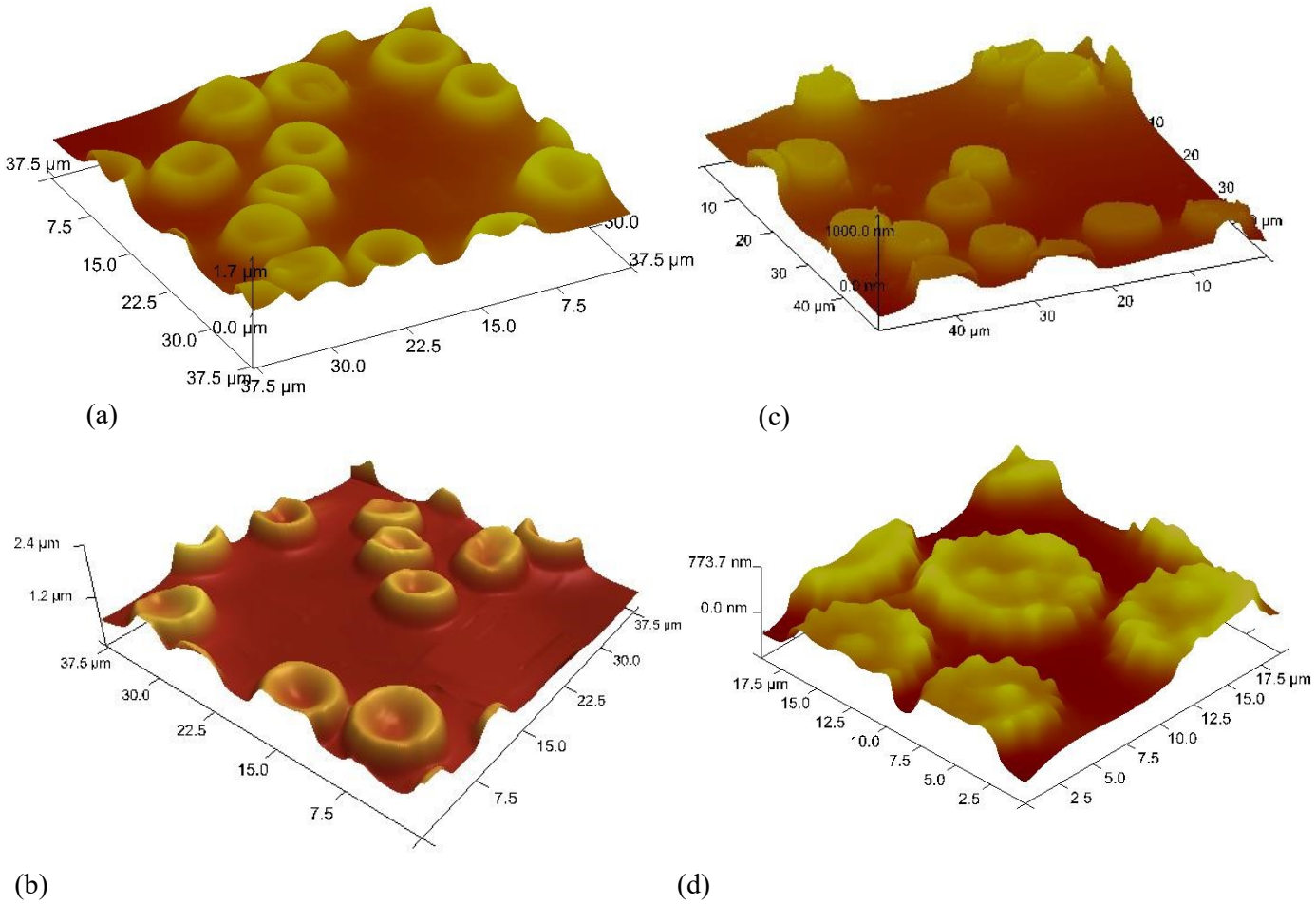

Fig. 1. 3D topography images of RBCs with AFM: (a) live cells, (b) live cells, c) early stage of ‘dying’ cells, d) later stage of ‘dying’ cells.

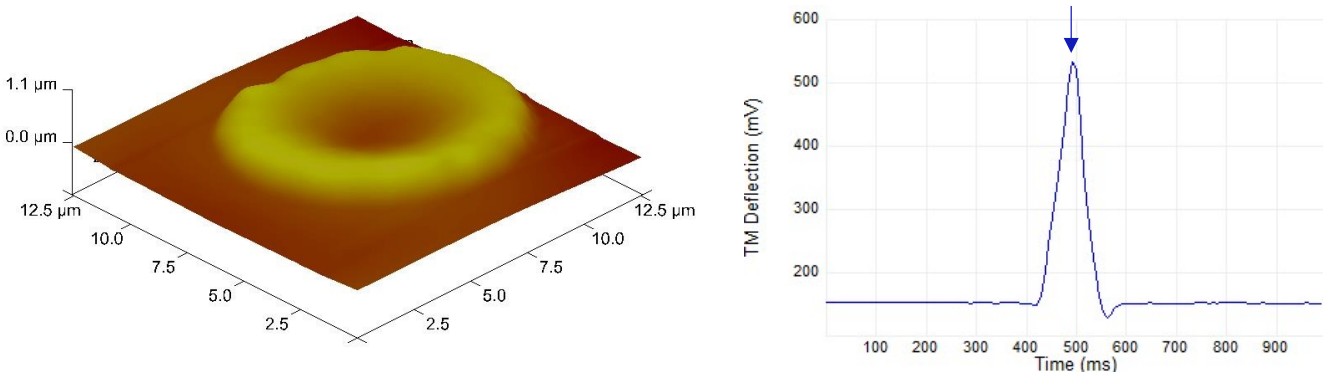

Fig. 2. (a) Single RBCs indented on membrane. (b) The change of DFL signal recorded on the RBC membrane during AFM indentation. The arrow indicates the rate-jump point across which Eq. (1) is applied to evaluate the elastic modulus of the cell.

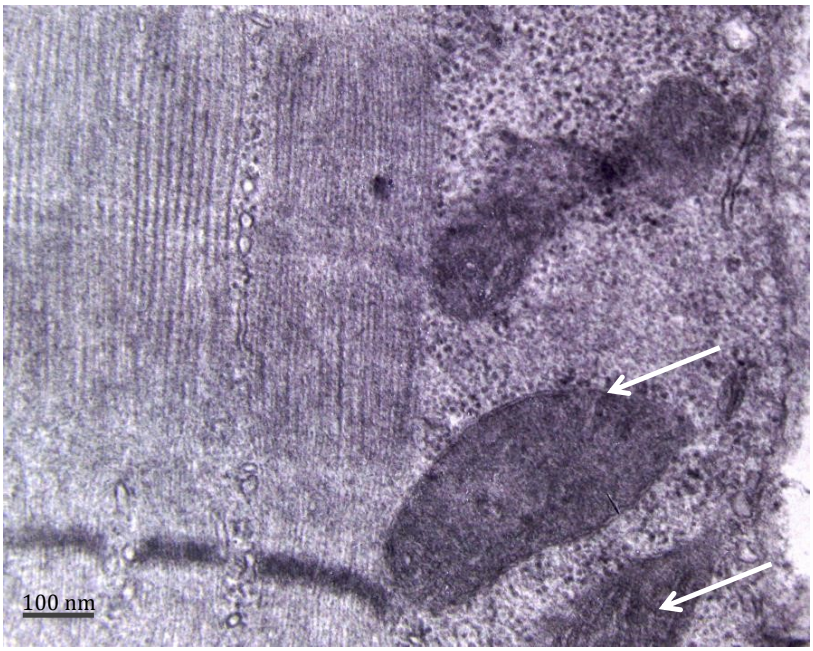

Fig. 3. Electron micrograph indicating increased mitochondrial volume and cristae density in the major gluteus muscle of a Lewis rat (AgB-1) treated with 2 mg of pentoxifylline for two weeks. The top arrow indicates the mitochondria, while the lower one their cristae.