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Original Article

Sildenafil citrate (Viagra) reduces surface roughness of human erythrocytes: Atomic-force-microscopic study

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ABSTRACT

Sildenafil citrate (Viagra) is used to treat erectile dysfunction and pulmonary arterial hypertension. The objective of this study was to analyze the action of sildenafil citrate on normal human erythrocytes *in vitro* at a concentration (2.5 mg/mL) higher than the prescribed for clinical conditions. Imaging of drug-treated erythrocytes was done using an atomic-force microscope in contact mode in air. Data analysis was performed using the scanning-probe-microscopy software WSxM. The study revealed that the drug causes hemolysis of erythrocytes at high concentration *in vitro* at room temperature. The ghosts (membranes) of erythrocytes with reduced cell size and deformed shape were observed using atomic-force-microscope imaging at low magnification. In addition, the high-magnification images revealed alterations in the nanostructural features of the erythrocyte membrane. There was a complete loss of characteristic membrane-architecture pattern. The root-mean-square surface roughness of the cell membrane after drug treatment was measured and found to be significantly less than that of erythrocytes in the native state. Sildenafil citrate causes hemolysis of erythrocytes *in vitro* at high concentration with significant alterations in morphometric properties, like change in cell shape, reduction in cell dimension, and disruption of membrane cytoarchitecture, along with a severe drop in membrane root-mean-square surface roughness.

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1. Introduction

The atomic-force microscope (AFM), since its invention in 1986 by Binnig et al [1], has become a powerful technique to image surfaces in material sciences. Within a few years, in 1993, DNA was efficiently imaged by AFM [2]. Since then, with significant efforts in improvisation of instrument setup as well as sample-preparation techniques, many reports concerning the application of AFM study of biological samples have been published. The surface topography of erythrocytes has been vastly studied by AFM by many researchers [3–7] since Zhang et al in 1995 [3]. Guha et al in 2002 [6] reported the characteristic ultrastructure pattern of human erythrocyte membrane consisting of "holes" and "blebs" of defined dimensions. This pattern is conserved in the hierarchy of species ranging from fish to mammals [7].

Ultrastructural studies with AFM are useful in evaluating parameters, like membrane surface roughness or power spectral density, which gives a quantitative measurement of the nanostructural features of the cell membrane. Surface roughness determines the texture of a surface, which is comprised of elevations and depressions. Many reports concerning surface-roughness calculations have been published [8,9]. The most popular means to assess the texture of a cell surface is to calculate the root-mean-square (rms)

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roughness of the cell. The mathematical formula represents the summation of deviation of heights of surface particles from the mean height in a selected surface extend. The formula is as follows:

$$R_{rms} = \sqrt{\sum_{i=1}^{N} \frac{(Z_i - Z_m)^2}{(N-1)}}$$

where N is the total number of data points, z_i is the height of the ith point, and z_m is the mean height. This parameter is scale dependent; it depends on the scan area and the number of data points [10]. The cell surfaces are involved in important phenomena, like adhesion, motility, and intracellular contact [11–13], making it worthwhile to quantify surface roughness.

Drugs enter the blood circulation after being absorbed in the intestine. The drug molecules may reach the site of action by adhering to the erythrocytes. The binding interaction between the erythrocytes and the drug molecules may lead to certain changes in cell surface features. Sildenafil citrate is increasingly being prescribed for treating pulmonary arterial hypertension (PAH) [14] and erectile dysfunction (ED) [15].

The effect of sildenafil citrate in treating both ED and PAH is due to a common pathway of accelerating the downstream effects of nitric oxide (NO)-mediated signaling and vasodilation. Sexual stimulation causes the release of NO in the corpus cavernosum of the penis, which binds to receptors of the enzyme guanylate cyclase [16–18]. Guanylate cyclase causes the synthesis of the messenger, cyclic guanosine monophosphate (cGMP). The drug alters the signaling pathway associated with penile erection during sexual intercourse. This signaling molecule leads to the relaxation of smooth muscles of the penis and vasodilation, thereby increasing the blood flow and causing erection. There is a regulatory mechanism involving an enzyme, phosphodiesterase enzyme 5 (PDE5), which controls the blood flow in the penis during erection [19]. It regulates the process by degrading cGMP and inhibiting signaling cascade responsible for the erection. Sildenafil citrate is analogous to cGMP and a competitive inhibitor of PDE5. It competes for binding of cGMP to PDE5 in the corpus cavernosum of the penis, resulting in less degradation of cGMP molecules and a better inflow of blood. It is effective in treating PAH by the similar mode of action. NO is produced by NO synthases located in the vascular endothelial and airway epithelial cells. Similar downstream signaling via cGMP stimulates dilatation of vascular smooth muscle at both the arterial and venous levels. It causes vasodilation and relaxes the wall of the pulmonary artery carrying deoxygenated blood to the lungs from the heart, leading to decreased pulmonary arterial pressure [20,21]. Here, the target of the drug is the regulatory enzyme, PDE5, distributed within the vascular smooth muscles [22].

The present study aimed to evaluate the hemolytic action of sildenafil citrate on normal human erythrocytes *in vitro* at high concentration using AFM, and to determine morphometric parameters, like cell dimensions, nanostructure dimensions, and RMS roughness computed by the WSxM software, both prior to and after drug treatment.

2. Materials and methods

Blood samples were collected in EDTA vials from the healthy volunteers at the MGM Medical College and Chacha Nehru Bal Chikitsalaya Avam Anusandhan Kendra, Indore, (Madhya Pradesh), India. Written permission was obtained from the Head, Department of Paediatrics of the institute. Informed consent was obtained from the volunteers or their guardians/parents prior to blood collection. The participants selected for the study were healthy volunteers present at the institute who were either the staff of the department or guardians/parents accompanying their wards for any clinical problem. The experiment was repeated on blood samples collected from five healthy donors.

Sildenafil citrate is marketed under the trade name Viagra for the treatment of ED by oral administration of tablets equivalent to 25 mg, 50 mg, and 100 mg. The prescribed dose for ED is 50 mg initial and 25–100 mg maintenance dose, once a day, at least an hour prior to the sexual activity. The drug is marketed with the name Revatio RVT 20 for treating PAH. The dose for treating PAH is 5 mg or 20 mg thrice a day with 20 mg as the maximum dose. The dose selected for the experiment was 2.5 mg/mL, much higher than the prescribed dose for treatment of either ED or PAH, so that its effect on erythrocytes and their membrane ultrastructure can be evaluated.

Blood was diluted with normal saline in 1:4 ratios to obtain diluted blood with an effective packed cell volume (PCV) of 8%. The sildenafil-citrate powder was acquired from the Quality Control Department of Cipla Pharmaceuticals, Indore, India. Fifty milligrams of powder were dissolved in 10-mL saline (0.09% NaCl solution) to obtain a 5 mg/mL solution. This solution was diluted 1:1 to obtain 2.5 mg/mL sildenafil-citrate solution. Two milliliters of 5 mg/mL sildenafil-citrate solution was added to 2-mL diluted blood (PCV 8%). The effective concentration of the drug becomes 2.5 mg/mL at PCV 4% [23]. Four milliliters of the resultant suspension was incubated for 90 minutes. Two milliliters of the diluted blood sample was further diluted with normal saline to obtain 4% PCV, which was a control. After the blood samples were given drug treatment and incubated for the determined time duration, the tubes were centrifuged at 1500 rpm and the supernatant was visibly checked for its color. The color of the supernatant in the experimental tube was compared with that of the control tube. The supernatant was discarded and pellets were suspended in 4-mL normal saline. The glass slides were cleaned properly and labeled, and then thin blood smear was drawn on them. The slides were air dried and cut into 1 cm × 1 cm pieces. The pieces containing the blood sample were mounted on sample holder with the help of a double-sided adhesive tape. The sample holder was loaded on the stage of the AFM for AFM imaging in real time [6]. The AFM facility was used at UGC-DAE Consortium for Scientific Research, Khandwa Road, Indore, India. The instrument used for the imaging was NanoScope E series scanning probe microscope (Digital Instruments, Santa Barbara, CA, USA) with standard top view. The images were captured in real time in contact mode, and analyzed using the WSxM software version 3.1.

3. Results

3.1. Hemolysis of erythrocytes

Treatment of normal human red blood cell (RBC) *in vitro* with sildenafil citrate at 2.5 mg/mL concentration for 90 minutes caused complete hemolysis, as evident by the red-colored supernatant obtained after centrifugation of the drug-treated blood sample. No hemolysis was observed in the tube, which was incubated for 30 minutes after drug treatment. The supernatant collected from the control tube after centrifugation was clear, indicating no sign of hemolysis.

3.2. Disruption of cell shape and dimension

A cluster of normal RBCs is imaged by AFM at low magnification, as seen in Figure 1A. The dark central region represents the concavity of the cell, while the peripheral light region represents the periphery of the disk-shaped biconcave cell. The section analysis of the cell indicates that the diameter of the highlighted RBC in Figure 1A is 7.82 μ. The AFM image at low magnification of normal human erythrocytes treated with sildenafil citrate at 2.5 mg/mL concentration for 90 minutes can be seen in Figure 1B. The discoid shape of normal erythrocytes (Figure 1A) appears distorted due to hemolysis, as seen in Figure 1B. The cell membrane is uneven, and is more or less fragmented showing sharp undulations. The cell size is reduced to 2.129 μ.

3.3. Alteration in membrane ultrastructure

The ultrastructure of normal RBC membrane in native condition shows the presence of "holes" surrounded by blebs in high-magnification AFM micrograph, as shown in Figure 2A. The high-magnification image of the drug-treated cells shows the disappearance of holes and blebs, as evident in Figure 2B. There is complete loss of characteristic membrane pattern, and granules of varying sizes and shapes can be observed. The sizes of granules vary from 20 nm to 40 nm. The membrane appears unevenly granular, possibly due to the denaturation of membrane protein moieties.

3.4. Reduction in RMS surface-roughness analysis after treatment with sildenafil citrate

The RMS surface-roughness value was computed using the WSxM software. The distribution of roughness value measured on the surface extend of 1 × 1 μm^2 of a normal erythrocyte and drug-treated erythrocyte is shown in Figures 3A and 3B, respectively. The former has a higher RMS roughness value (23.113 nm) as compared to the latter (14.811 nm). The Student *t* test for dependent variables was conducted, and there was a significant difference between the scores for the roughness of normal RBCs (M = 21.42, standard deviation = 3.24) and the sildenafil-citrate-treated RBCs (M = 12.6, standard deviation = 1.39); $t(4) = 5.73$, $p < 0.05$.

4. Discussion

Sildenafil citrate is considered an important drug due to its widespread action on a number of disorders. Its role as an agent for the prevention and treatment of high-altitude sickness suffered by mountain climbers has been documented [24]. Jet-lag recovery has also been attributed to sildenafil citrate [25]. Vaginal application of sildenafil increases the endometrium thickness in women with history of recurrent miscarriage, and also it has been reported to decrease natural-killer-cell activity in such women [26]. The most important action is to enhance performance during a sexual activity.

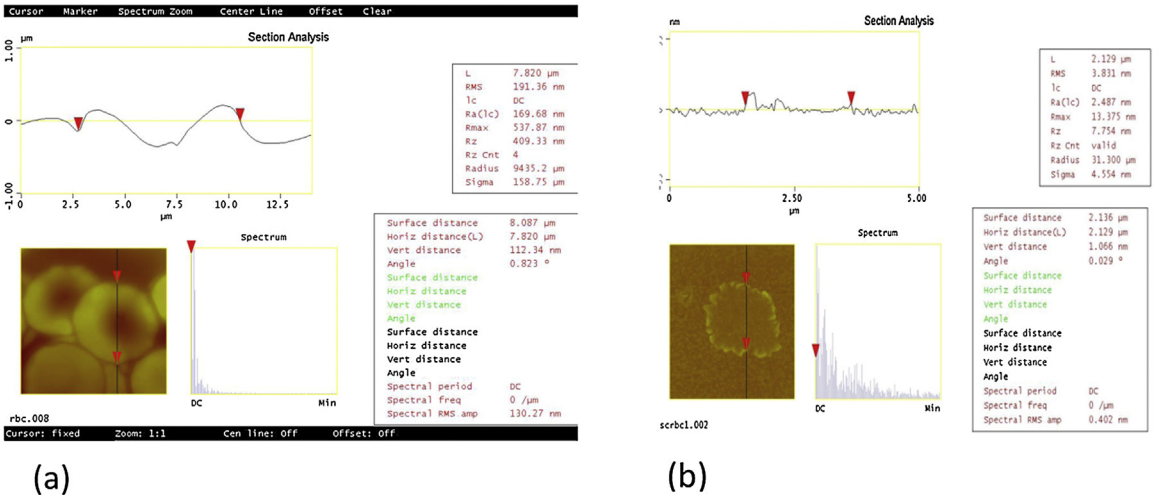

Fig. 1. (A) Section analysis of human red blood cells in normal condition (low magnification). Scan size 15 μm. The figure shows an atomic force micrograph of a cluster of normal red blood cells. The section analysis shows that the diameter of the highlighted red blood cell is 7.820 μm. However, the surface distance representing the curved membrane defining the cavity is measured 8.087 μm. (B) Section analysis of sildenafil-citrate-treated normal red blood cell (low magnification). Scan size 5 μm. The figure shows ghost of red blood cell after treatment with 2.5 mg/mL sildenafil citrate for 90 minutes. Red blood cell appears completely hemolyzed. The cell size is 2.129 μm and the surface distance is 2.136 μm.

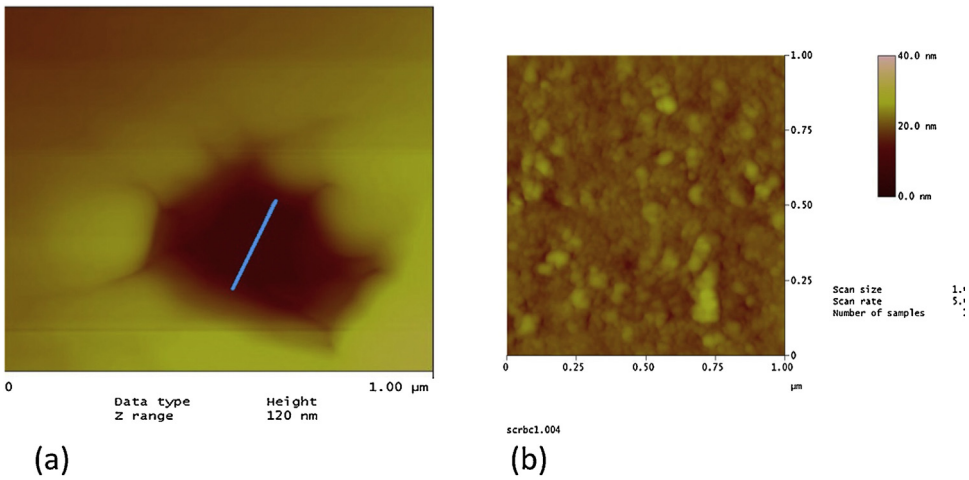

Fig. 2. Section analysis of red blood cell in normal condition (high magnification). Scan size 1 m. The figure shows high-magnification image of normal red blood cell. The characteristic ultrastructure of the cell membrane shows the presence of a "hole" surrounded by blebs. The diameter of the hole is 274 nm. (B) Section analysis of sildenafil-citrate-treated normal red blood cell (high magnification). Scan size 1 m. The figure shows membrane cytoarchitecture of normal red blood cell after treatment with sildenafil citrate at 2.5 mg/mL concentration for 90 minutes. The membrane appears granular. The sizes of granules range from 20 nm to 40 nm.

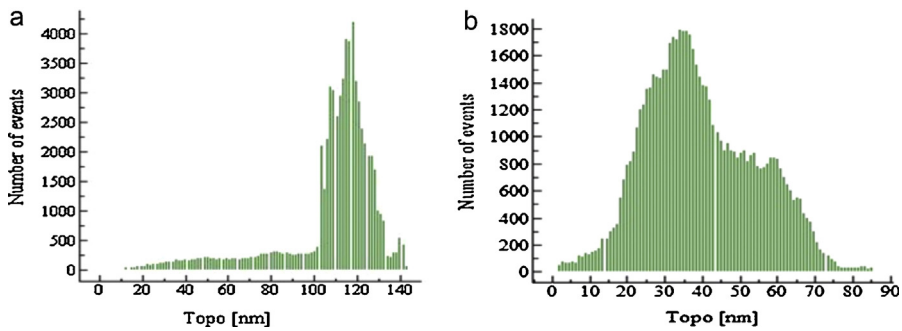

Fig. 3. Distribution of roughness value measured on the surface extend of $1 \times 1\ \mu m^2$ of (A) a normal erythrocyte and (B) sildenafil-treated erythrocyte. *X* axis: particle height (nm); *Y* axis: number of events; number of points – 100. Root-mean-square roughness: (A) 23.113 nm and (B) 14.811 nm.

The use of AFM to evaluate the *in vitro* action of sildenafil citrate on erythrocytes stems from its potential to quantify morphological features with utmost precision. Many reports concerning AFM study of the action of drugs on normal erythrocytes have been published.

Zuk et al in 2011 [27] published a report on the study of the effect of a few selected drugs used in asthma treatment on the morphology and elastic properties of RBCs. Greater sizes of cells were noticed on the treatment with aminophylline at a low concentration ($8.3 \pm 0.1\ \mu m$ in diameter), whereas not much change could be noticed in the case of methylprednisolone treatment at a low concentration. However, the stiffness of RBCs incubated with both drugs was found to be increasing with the increase in incubation time, which may result in a reduced capability of the cells to carry oxygen molecules.

The action of a local anesthetic drug called procaine on erythrocytes was studied by Zdrenghea et al in 2011 using AFM [28]. The drug treatment resulted in changes in cell concavity depth and surface roughness of erythrocyte membrane. Also, the particles on the membrane surface increased with increasing procaine concentration. They reported the presence of a large number of elevations of the order 20–40 nm on the erythrocytes treated with low and medium procaine concentrations. The granules measuring 80–90 nm were noticed arranged in row in high-procaine-concentration-treated erythrocytes.

The antimicrobial properties of analgesic kyotorphin peptides on erythrocyte membrane have been investigated using AFM [29]. The AFM study of the action of drugs on erythrocytes is also efficacious in studying the permeation properties of cell membrane. Recently, the action of lanthanide cation, gadolinium, was studied on erythrocyte membrane by Cheng et al [30]. The results revealed an enhanced permeability of the membrane. Two different modes of perforation were noticed, the domain structure and the pore structure, depending upon the gadolinium concentration.

Aluminum is an element toxic to the human body. The study of the effect of aluminum concentrations on RBC membranes revealed physicochemical modifications of erythrocytes at the membrane level. Aluminum-induced lipid peroxidation reduced the activity of erythrocyte antioxidant enzymes. Aluminum-induced morphological

changes were studied using atomic-force microscopy [31]. Chernysh et al [32] reported ultrastructural changes in erythrocyte membranes after treatment with zinc ions at varying concentrations. However, restoration of membrane ultrastructure was noticed by the addition of albumin. Similarly, another AFM investigation demonstrating the effects of zinc ions at different concentrations on the nanostructure of RBC membranes, *in vitro*, revealed hemoglobin aggregation and the presence of altered conjugate processes on RBC membranes at a high concentration (0.5 ± 0.1 mM) [33]. There was a significant decrease in the membrane roughness of erythrocytes of smokers when compared with nonsmokers [34].

Systemic research is affected by the problem of intoxication of blood. The process of intoxication leads to alterations in the ultrastructure of cell membranes [35,36]. Intoxication may result in leakage of hemoglobin from RBC to blood plasma in conditions like intravascular hemolysis, hemorrhagic shock, hemolytic anemia, transfusion of incompatible blood, and other pathological conditions. The products of hemoglobin destruction, in particular free hemin, lead to endogenous intoxication [37]. The elasticity of RBC has been found to be decreased in the case of exogenous intoxication with hemin [38]. Heavy-metal ions and medicines in high doses may also cause intoxication. Furosemide at high dose acts as an inhibitor of anion transport through band 3 [39]. The treatment of RBC with chlorpromazine at high concentration can cause clustering of integral membrane proteins, in particular band 3 [40,41].

Drugs, like tunicamycin, have also been reported to show RBC membrane active property. It is a nucleoside antibiotic detrimental to the growth of a number of viruses, protozoans, and metazoans due to its role in the intervention of glycosylation of glycoprotein. The drug has been found to have an effect on rabbit erythrocytes [23]. This effect is concentration dependent. At a low concentration, the normal discocyte changes to echinocyte, which finally transforms to a spherocyte at a higher concentration.

The hemolytic action of sildenafil citrate on normal erythrocytes by *in vitro* experiment can be observed from the cell morphology. Roughness analysis supported the observations derived from AFM images. A decrease in surface roughness may be related to the action of sildenafil citrate on the cytoskeleton network of the cell. A similar report was published by Girasole et al in 2007 [42], where discocytes with a higher RMS roughness value treated with cytochalasin D showed a reduction of roughness possibly due to the disruption of the skeletal network.

The anchoring proteins on the cell membrane are connected to the cytoskeleton proteins by actin and spectrin to impart a proper shape to the cell. It may be assumed that these anchoring proteins on the cell membrane may contribute to the cell surface roughness. Sildenafil citrate might interact in the outer layer of the protein lipid bilayer, and as a result, the anchoring proteins contribute less to the surface roughness. A decrease in surface roughness may be related to the action of sildenafil citrate on the cytoskeleton network of the cell.

Although treatment with sildenafil citrate has been observed to cause hemolysis of erythrocytes *in vitro*, the direct extrapolation of our results as contraindications is limited because our investigations are being carried out *in vitro* at a high concentration of drugs. The usage of sildenafil citrate for treating PAH is generally limited, but its use (repeated and high dose) for recreation purpose for enhancing sexual performance, without prescription, might pose threats to consumers suffering from nutritional-deficiency anemia or any type of hemoglobinopathy. Our observations reported here are of general scientific importance, which can find relevance to further research regarding the ultrastructure of cell membranes and the biological actions of chemically similar drugs, like tadalafil.

Conflicts of interest

The authors have no conflicts of interest to declare.

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