Journal of Microscopy and Ultrastructure 4 (2016) 63–68

Contents lists available at ScienceDirect

Journal of Microscopy and Ultrastructure

journal homepage: www.elsevier.com/locate/jmau

Original Article

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

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ARTICLE INFO

Article history:
Received 19 May 2015
Received in revised form 26 October 2015
Accepted 28 November 2015
Available online 14 December 2015

Keywords:
Atomic-force microscope
Erythrocyte
Root-mean-square surface roughness
Sildenafil citrate

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.

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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http://dx.doi.org/10.1016/j.jmau.2015.11.005
2213-879X/

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.