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Brief Communication

Smoking and fluidity of erythrocyte membranes: A high resolution scanning electron and atomic force microscopy investigation ☆

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ABSTRACT

Smoking affects the general health of an individual, however, the red blood cells (RBCs) and their architecture are particularly vulnerable to inhaled toxins related to smoking. Smoking is one of the lifestyle diseases that are responsible for the most deaths worldwide and an individual who smokes is exposed to excessive amounts of oxidants and toxins which generate up to 10^{18} free radicals in the human body. Recently, it was reported that smoking decreases RBC membrane fluidity. Here we confirm this and we show changes visible in the topography of RBC membranes, using scanning electron microscopy (SEM). RBC membranes show bubble formation of the phospholipid layer, as well as balloon-like smooth areas; while their general discoid shapes are changed to form pointed extensions. We also investigate membrane roughness using atomic force microscopy (AFM) and these results confirm SEM results. Due to the vast capability of RBCs to be adaptable, their state of well-being is a major indication for the general health status of an individual. We conclude that these changes, using an old technique in a novel application, may provide new insights and new avenues for future improvements in clinical medicine pertaining to conditions like COPD.

Introduction

Smoking is one of the lifestyle diseases that cause the most deaths worldwide. An individual who smokes is exposed to excessive amounts of oxidants and toxins which generate up to 10^{18} free radicals to the human body [1–4]. In 1989, Hannan and co-workers showed that cigarette smoking causes a decrease in membrane fluidity of rat alveolar macrophages [5]; and in 1992 it was suggested that superoxide radicals or subsequently generated species contained in the gas phase of cigarette smoke increases the intracellular water organization in Jurcat cultured T cells [6]. A year later, in 1993, it was shown that superoxide generated by cigarette smoke also damages the respiratory burst and induces physical changes in the membrane order as well as water organization of lymphocytes in culture [7].

It was also shown that in cigarette smoke exposed buffer solutions, superoxide ($O_2{\cdot}-$) was the predominant generated reactive oxygen species [8]. This was demonstrated by lucigenin-amplified chemiluminescence and electron spin resonance (ESR) spin-trapping with 5,5-dimethyl-1-pyrroline N-oxide (DMPO). In a study by Tsuchiya and co-workers in 2002, the authors showed that smoking a single cigarette temporarily decreases nitrate, nitrite, and serum antioxidant concentrations in the plasma [9].

More recently, research in humans focussed on the effect of toxins in cigarette smoke and confirmed severe oxidative stress, when inhaled, resulting in inflammation [1]. Cell membranes are particularly vulnerable to oxidative stress and play a major role in disease pathology and progression. Phospholipids form a major constituent of membranes and therefore the properties and condition of biological membranes are a function of their lipid composition [10].

Previous research has shown alterations in platelet membrane fluidity during smoking [2]; and the reason for this was noted to be due to an increase in lipid peroxidation and carbonyl groups. Changes to the lipid bilayer and damage to polyunsaturated fatty acids were suggested to cause the decreased fluidity [2]. Pretorius in 2012 confirmed these changes by showing that platelet membrane fluidity changes can be seen by using scanning electron microscopy [11].

In 2012, researchers for the first time also showed that smoking causes a decrease in membrane fluidity and possibly impair the functions of the plasma membranes of red blood cells (RBCs) in patients with COPD [10]. Research suggested that RBC membrane

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lipids are rich in polyunsaturated fatty acids; and therefore the exposure to toxins and peroxidants, due to cigarette smoke, causes haemolyses as well as loss of membrane stability [12–14].

In the current investigation we investigate changes in RBC membrane structure and show these changes in smokers, using scanning electron microscopy (SEM), as well as atomic force microscopy (AFM). We also show that superoxide was significantly higher in whole blood of the smoker group. Furthermore, we show the extent of the RBC membrane changes and argue that this has greater implications for general health than previously thought.

Materials and methods

Sample

The sample consisted of 30 smokers (male and female) between the age of 20 and 60, who did not suffer from high blood pressure or heart conditions and who classified themselves as healthy individuals. None of the individuals used any medication or products other than smoking. Smokers smoked on average five cigarettes per day for more than 5 years (see Table 1 for data on sample). Micrographs from smokers were compared to healthy individuals between the ages of 20 and 60 who did not smoke; as well as to our micrograph database of thousands of RBCs from healthy individuals. The population of healthy individuals did not use any medication or in the case of the females, use contraception or hormone replacement therapy. Ethical clearance was obtained for this study from the University of Pretoria Human Ethics Committee.

Whole blood smear preparation for scanning electron microscopy

Whole blood smears to study RBCs from participants were prepared by making a smear on a glass cover slip. The glass cover slips were placed in a Petri dish on filter paper dampened with phosphate buffered saline (PBS) to create a humid environment and placed at 37 °C for 10 min. A washing process where samples were placed in PBS and stirring for 20 min followed this. PRP smears were fixed in 2.5% glutaraldehyde/formaldehyde in Dulbecco's phosphate buffered saline (DPBS) solution with a pH of 7.4 for 30 min. The samples were rinsed 3× in phosphate buffer for 5 min before being fixed for 30 min with 1% osmium tetra-oxide (OsO_4) This was followed by another 3× rinsing with PBS for 5 min each time, followed by a serial dehydration, in 30%, 50%, 70%, 90% and three times with 100% ethanol. The material was dried, mounted and coated with carbon. A Zeiss ULTRA plus FEG-SEM with InLens capabilities were used to study the surface morphology of erythrocytes and micrographs were taken 1 kV accelerating voltage. This instrument is located in the Microscopy and Microanalysis Unit of the University of Pretoria, Pretoria, South Africa.

Sample preparation – AFM

1 ml of blood was drawn in citrate tubes and centrifuged at 1000 rpm for 2 min. The supernatant was discarded (plasma, platelets and white blood cells) and the remaining pellet (erythrocytes) was suspended in 2.5% glutaraldehyde/formaldehyde in Dulbecco's phosphate buffered saline (DPBS) solution with a pH of 7.4 for 30 min, rinsed with DPBS and post-fixed with OsO4 to ensure the preservation of membrane phospholipids. The samples were dehydrated with a series of ethanol and dried on a glass coverslip using HMDS.

AFM imaging and measurement

An AFM (Dimension Icon, Bruker, USA) was used in tapping mode to obtain topographic images. Aluminium coated nitride tips (TESPA, Bruker, USA) with a spring constant of 20–80 N/m, a resonant frequency between 382 and 405 kHz and a nominal tip radius of 8 nm was employed in all AFM measurements. Six cells of each sample were scanned at a 1 by 1 μm scan size. NanoScope Analysis (Bruker, USA) was used to filter out any noise, subtract the plane of average inclination and perform all measurements.

Membrane roughness was determined by using power spectral density which provides a representation of the amplitude of a surface's roughness as a function of the spatial frequency (by Fast Fourier Transform) of the roughness. This allows one to specify spectral thresholds at predetermined frequencies and measuring the root mean square (RMS) roughness of the specific spectral window. Three spatial domains were isolated, each reflecting a typical erythrocyte membrane feature as shown in literature [15,16]. The statistical significance of the difference between measurements was determined using one-way analysis of variance. A 2-tailed P value of less than 0.05 was considered significant.

Flow cytometry of whole blood superoxide levels

HE detects superoxide ($O_2\bullet-$) within the living cell and it is enzymatically dehydrogenated, in part, to form ethidium, which becomes locked in the cell by virtue of its cationic nature. It is excited by visible 535 nm light. Fig. 3 shows the levels of superoxide in whole blood of healthy individuals versus that of smokers. These results confirm previous research, suggesting that superoxide generation is significantly higher in smokers.

Results

SEM analysis

Fig. 1 A and B shows a typical erythrocyte (red blood cell – RBC) at a low SEM (25000× machine magnification) and a high 100,000× machine magnification showing the membrane ultrastructure. The membranes of healthy RBCs have a typical globular architecture. Fig. 1C and D show RBC micrographs from a smoker. The lower magnification (Fig. 1C) demonstrates a general shape change in smoking, where the RBC deform from the typical discoid shape to form pointed extensions. Pretorius and co-workers have recently reported similar shape changes in inflammatory conditions like thrombo-embolic ischemic stroke, diabetes and in iron overload [17]. Importantly, this shape change is not visible under low magnifications that can be obtained by light microscopy. Also, surface membrane changes in smoking have been noted here for the first time at machine magnification of 100,000× (Fig. 1D).

AFM analysis

Table 2 shows the roughness measurement results of healthy individuals versus smokers.

Table 1
Data on healthy individuals and smokers (all within a healthy body mass index (BMI)).

Individual	Males	Females	Age range from 18 to 22	Age range from 30 to 50	Age range from 51 to 65
Healthy individuals	15	15	20	5	5
Smokers	15	15	20	5	5