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QUALITATIVE AND QUANTITATIVE ASSESSMENT OF OXIDATIVE STRESS IN HUMAN LIVING ERYTHROCYTES USING TWO-PHOTON MICROSCOPY IMAGING TECHNIQUE

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Red blood cells (RBCs) are unceasingly exposed to exogenous reactive oxygen species (ROS) during their passage through the circulatory system. Nevertheless, RBCs have a large antioxidant system including ascorbate, vitamin E, catalase, superoxide dismutase, ergothioneine, glutathione, glutathione peroxidase and peroxiredoxin, which are assumed to eliminate ROS. The purpose of this study is to design a new effective method for evaluation of oxidative stress in human living RBCs using two-photon microscopy imaging. For imitation of oxidative stress in human living RBCs, an *in vitro* model was created and imaged by two-photon microscopy. The results showed that oxidative stress is obvious on the two-photon microscopy images of RBCs under oxidative stress in comparison with the absence of fluorescence in control samples ($P<0.05$). This new approach for estimation of oxidative stress in human living RBCs could be successfully applied in various fields of clinical research and analysis of antioxidant components.

Oxidative stress – red blood cells – two-photon microscopy

Կարմիր արյան բջիջները (ԿԱԲ) շրջանառության համակարգով իրենց անցման ընթացքում անդադար ենթարկվում են էկզոգեն թթվածնի ակտիվ ձևերի (ԹԱՁ) ազդեցությանը: Այնուամենայնիվ, ԿԱԲ-ներն ունեն մեծ հակաօքսիդանտային համակարգ, որը ներառում է ասկորբինաթթու, վիտամին E, կատալազ, սուպերօքսիդ դիսմուտազ, էրգոթիոնեին, գլուտաթիոն, գլուտաթիոն պերօքսիդազ և պերօքսիռեդօքսին, որոնք, ենթադրաբար, հեռացնում են ԹԱՁ-երը: Տվյալ հետազոտության նպատակն է մշակել նոր արդյունավետ մեթոդ՝ մարդու կենդանի ԿԱԲ-ներում օքսիդատիվ սթրեսի գնահատման համար՝ կիրառելով երկֆոտոն մանրադիտարկման պատկերումը: Մարդու կենդանի ԿԱԲ-ներում օքսիդատիվ սթրեսի իմիտացիայի համար ստեղծվել է *in vitro* մոդել և պատկերվել երկֆոտոն մանրադիտակի օգնությամբ: Արդյունքները ցույց են տվել, որ օքսիդատիվ սթրեսի պայմաններում մշակված մեթոդով օքսիդատիվ սթրեսն ակնհայտ գրանցվում է ԿԱԲ-ների երկֆոտոն մանրադիտարկման պատկերներում՝ համեմատած ստուգիչ նմուշներում լուսարձակման բացակայության հետ ($p<0.05$): Մարդու կենդանի ԿԱԲ-ներում օքսիդատիվ սթրեսի գնահատման համար այս նոր մոտեցումը կարող է հաջողությամբ կիրառվել կլինիկական հետազոտությունների և հակաօքսիդանտային բաղադրիչների վերլուծության զանազան բնագավառներում:

Օքսիդատիվ սթրես – կարմիր արյան բջիջներ – երկֆոտոն մանրադիտարկում

Красные кровяные тельца (ККТ) во время прохождения через систему кровообращения постоянно подвергаются воздействию экзогенных активных форм кислорода (АФК).

Тем не менее, ККТ имеют большую антиоксидантную систему, включая аскорбат, витамин Е, каталазу, супероксиддисмутазу, эрготионеин, глутатион, глутатионпероксидазу и пероксиредоксин, которые, как предполагается, устраняют АФК. Целью данного исследования является разработка нового эффективного метода оценки окислительного стресса в живых ККТ человека с использованием двухфотонной микроскопии. Для имитации окислительного стресса в живых ККТ человека была создана модель *in vitro*, которую визуализировали с помощью двухфотонной микроскопии. Результаты показали, что окислительный стресс очевиден на изображениях двухфотонной микроскопии ККТ в условиях окислительного стресса по сравнению с отсутствием флуоресценции в контрольных образцах ($p<0.05$). Разработанный новый подход к оценке окислительного стресса в живых ККТ человека может быть успешно применен в различных областях клинических исследований и анализа антиоксидантных компонентов.

Окислительный стресс – красные кровяные тельца – двухфотонная микроскопия

Oxidative stress is connected to the enhancement of oxidizing agents' production and a sharp decrease in the effectiveness of antioxidants [28]. This can be a serious reason for the development of age-related human diseases, such as neurological diseases, ulcers, pneumonia, cataract, cancer, diabetes, rheumatoid arthritis, cardiovascular disorders, glaucoma and human aging [15, 25, 26].

Red blood cells (RBCs) are one of the first cells exposed to endogenous and exogenous reactive oxygen species (ROS) [2, 30]. But, at the same time, erythrocytes have strong antioxidant system which allows keeping the oxidant/antioxidant balance in the organism [18]. Nevertheless, various pathological states can lead to crucial changes in this antioxidant system and enzymes activity [1, 6].

That is why it is very important to study and evaluate the mechanism of oxidative stress in RBCs in disease states. There are many different published works devoted to study the effects of ROS in RBCs [9, 19, 27, 32, 33]. In pathological conditions, ROS-induced damage of RBC membrane compounds considered to increase erythrocyte membrane fragility and rigidity, followed by intravascular hemolysis, release of hemoglobin into the plasma and systemic nitric oxide scavenging [9]. Recently, a group of researchers conducted a very interesting work about the assessment of oxidative stress and the effects of ROS in critically ill patients with COVID-19 [19]. It was shown that excessive levels of ROS lead to the misbalance of neutrophil to lymphocyte ratio, tissue damage, thrombosis and RBCs dysfunction, as well as increase the severity of COVID-19 disease in these patients [19]. Most techniques for ROS study in RBCs consider measuring of the end product of oxidation or cell's antioxidant activity and use the products of RBCs lysis but not the cells [32, 33]. It is also significant to mention that various methods and ways have been designed for visualization of RBCs [3, 12, 24].

Nowadays, application of two-photon microscopy becomes more and more actual. Two-photon microscopy is a fluorescence imaging technique that allows imaging of living tissue up to a very high depth, about one millimeter. Being a special variation of the multiphoton fluorescence microscope, it uses red-shifted excitation light which can also excite fluorescent dyes. It is a brilliant research tool for investigation of biological samples, including cells, tissues, organs and even the whole organism due to its deeper tissue penetration, efficient light detection and reduced photo toxicity [7]. Two-photon microscopy has many applications in various fields, such as biomedicine, biochemistry, biophysics, biotechnology, microbiology, molecular biology, histology, drug design, material and life sciences, etc.

What concerns study the human living erythrocytes, two-photon microscopy is used for their optical trapping [22]. But experiments in this field are still limited.

Consequently, the main goal of present work is not only the analysis of RBCs using Two-photon microscopy imaging, but also the qualitative and quantitative assessment of oxidative stress which will open new perspectives for the development of new and effective anti-aging substances.

Materials and methods. *Experimental design.* Isolated human living RBCs were exposed to H_2O_2 to imitate oxidative stress. Two-photon microscopy imaging was performed by using a ROS-sensitive membrane-permeable fluorescent dye, 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA, Sigma-Aldrich Chemie GmbH, Germany).

Study subjects. A total of 20 healthy volunteers (mean age ± SD: 30 ± 9 years, females/ males: 10/10) from the Institute of Molecular Biology NAS RA were enrolled in this study. All subjects were healthy, active and living independently at home, reporting no serious medical disorder or treatment during the past 12 months. All subjects gave their informed consent to participate in the study, which was approved by the Ethical Committee of the Institute of Molecular Biology NAS RA *(IRB #00004079)***.**

Blood collection and erythrocyte isolation. Practically fasting blood samples (~2 ml each) were collected by venipuncture in EDTA containing tubes using plastic syringe and relatively wide-bore needle according to the WHO guidelines on drawing blood [37]. To isolate RBCs, immediately after blood collection the blood samples (200 µl) were washed twice in isotonic saline solution (0.9% NaCl) and once in isotonic phosphate-buffered saline (PBS; NaH2PO4·2H2O 123 mmol/l, Na2HPO4 27 mmol/l, NaCl 123 mmol/l; pH 7.4) at 2000 g at 4°C for 5 minutes. The blood components, plasma and buffy coat, were removed by aspiration after the first wash, leaving RBCs at the bottom of the centrifuge tube. After the washing procedures, a suspension of RBCs was prepared by adding PBS to the washed and aliquoted RBCs (RBC-PBS solution) to obtain 1 to 10 dilutions [31].

Generation of an in vitro model of oxidative stress. The RBC-PBS suspension was used as the negative control for oxidative stress. *In vitro* oxidative stress was generated by addition of peroxide to 40 µl of RBC-PBS solution (RBC-PBS-H_2O_2). After the incubation at 37°C for 40 minutes, the samples were washed twice in PBS under the above described conditions [34, 35].

Imaging of oxidative stress in RBCs by two-photon laser scanning microscopy. For the detection of oxidative stress inside living cells, the intact RBC-PBS and RBC-PBS-H_2O_2 samples were treated with a carboxy-DCFDA, most commonly used for detection of changes in redox state in a cell [4, 11]. While not excited, carboxy-DCFDA is colorless and non-fluorescent. However, upon cleavage of two acetate groups by intracellular esterases and conversion to fluorescent fluorophore, 5(6)-carboxy-2',7'-dichlorofluorescein, it emits bright green fluorescence proportional to ROS generation intensity. Ten µl of 100 µM carboxy-DCFDA diluted in DMSO were added to each sample with the subsequent incubation at 37°C for 30 minutes. After washing with PBS, the stained cells were resuspended in 40 µl PBS and were immediately prepared for two-photon microscopy imaging [34, 35].

Laser source. The laser source used for the two-photon scanning fluorescence microscopy is a diode-pumped Yb:KGW ultrafast oscillator ("t-pulse", Amplitude Systems, France) available at the AREAL facility [36]. The laser generates a high-repetition-rate (50 MHz) train of ultra-short (240 fs) pulses of quasi-monochromatic (~5 nm bandwidth) light at 1030 nm wavelength. The output average power of the oscillator is 1.1 W (energy per pulse ~22 nJ) which is too high for safe imaging of the samples. The power of the excitation is therefore regulated using a PC-controlled power attenuation kit placed in the beam path to maintain final power of 300 mW at the sample.

Two-photon fluorescence laser scanning microscopy system. The two-photon laser scanning upright microscope (MOM - Movable Objective Microscope, Sutter Instruments, USA) with 20× water immersion objective and numerical aperture of 1.0 and 2.0 mm working distance was used to capture microscopy images of RBCs.

Beam splitter mounted into laser beam path allows to change laser power at the surface of the samples in wide range (3-500 mW), which provides acquiring good contrast without detectable morphological changes in RBCs during the time of the experiment (30–60 min). The fluorescence of carboxy-DCFDA was detected using two-channel system with green (Full Width at Half Maximum (FWHM): 70 nm; Maximum transmission: 525 nm; Average transmision: 92%) filter

and a photomultiplier (R6357; Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany) with 185 – 900 nm bandwidth.

For the spatial investigation, *z*-stack image analysis of cells was performed with 30-45 cross-sections for each field of view in AVI Movie format with the z-scanning depth of 12 μm.

RBC images were obtained by x, y galvanometric scanner in standard (512x512 pixels; 3.05 fps frame rate) and hi-quality (1024x1024 pixels, 0.76 fps frame rate,) modes on 12 bits photomultiplier with pixel clock of 1000 ns and magnification of 4×. The images were false color coded for display. Image capturing and processing was conducted by the MOM microscope and Image J [29] software.

Image processing and data analysis. Image processing was performed using Fiji/Image J software (Image J 1.50i NIH, Bethesda, MD, USA) [29]. For the quantitative analysis of the cells fluorescence intensity in arbitrary units (AU) the acquired images were converted to 8 bit grayscale images. Afterwards, raw images were segmented by adaptive thresholding to facilitate automatic cell detection [17]. The 0-255 normalized scale was used for the selected images, where 0 corresponds to the black and 255 to the white color. The image intensity was calculated as a sum of intensities of all the cells from the ROI. Volume Viewer and 3D Viewer plugins were used for 3D deconvolution of two-photon microscopy *z*-stack images of cells [21].

Statistical analysis was performed using "GraphPad Prism 3.03" (GraphPad Software Inc., USA). The paired Student *t*-test was used to compare two conditions (with and without oxidative stress) using the original data. Groups' statistics is presented in dot plot graph. The 95% confidence interval and Pearson's value (*P*-value) were calculated to evaluate the effects of any difference. *P*-values less than 0.05 were considered statistically significant.

Results and Discussion. In the current study a novel modern approach of two-photon microscopy imaging was described for the *in vitro* analysis and evaluation of oxidative stress in human living RBCs. Erythrocytes were isolated from the blood samples of healthy volunteers and were used for the main investigation. RBCs were exposed to hydrogen peroxide for the *in vitro* imitation of oxidative stress. To estimate the intensity of oxidative processes in RBCs carboxy-DCFDA and two-photon laser scanning microscopy were used.

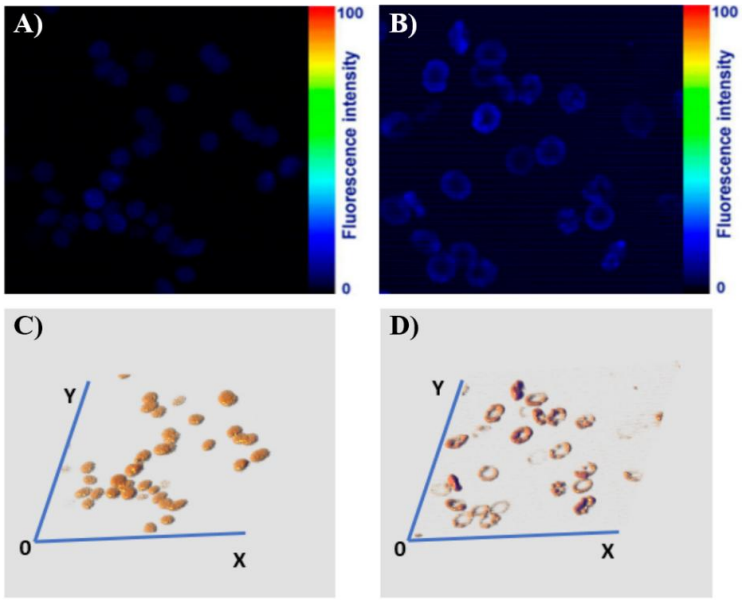

Fig. 1. Two-photon fluorescence intensity images of carboxy-DCFDA treated RBCs before (A) and after (B) the H_2O_2 exposure, as well as their 3D deconvolution z-stack images (C and D).

Before the main experiments, visualization of intact RBC samples was performed to ensure the presence of normal biconcave disk-shaped cells in samples. By the imaging of negative samples (without oxidative stress or H_2O_2) we ensured that we have normal RBCs with a shape of biconcave disk, but not crenated or spherical. Samples that primarily showed variations in the RBC morphology were not processed further. These were 2 samples initially demonstrating crenated RBCs that were excluded (fig. 1).

Thus, one of the negative effects of oxidative stress on erythrocytes is the obvious modification of their morphology. The change of morphology can be caused by oxidation of membrane lipids and proteins. The similar results have been obtained by another group of researchers that confirmed that morphologically abnormal erythrocytes were significantly correlated with oxidative stress and chronic inflammation markers. Due to increased oxidative stress the percentages of biconcave cells were decreased [14].

The application of two-photon microscopy imaging technique for the study of oxidative stress in human living RBCs highlights the novelty and specificity of the present work. Nowadays, investigations of oxidative stress in RBCs are very important to deeply understand the pathological states of the organism and also to estimate its antioxidant ability. Therefore, this novel approach can be very useful not only for the study of various diseases' mechanisms, but also for the assessment of the effects of different drugs, extracts, agents and irradiation on human living RBCs.

It was also important to count the cells in intact RBCs and RBCs after the H_2O_2 exposure. The results showed crucial changes in number of cells in RBCs. The cell count in intact RBCs was more than $4*10^6$, whereas after the H_2O_2 exposure the number of cells was significantly decreased (Fig. 2). Obtained results prove the fact that during oxidative stress, excess free radicals can damage structures inside cells and even cause cell death.

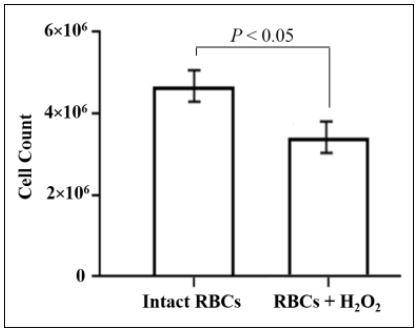

Fig. 2. Cell count in the samples of RBCs before and after the H_2O_2 exposure.

Similarly, the percentage of living cells notably varies between the intact RBCs and the RBCs after H_2O_2 exposure (tab. 1).

Various age-related human diseases are generally caused by oxidative stress [1, 2, 6, 15, 19, 25-27]. ROS are generated as a natural byproduct of the normal aerobic metabolism of oxygen and play important roles in cell signaling and homeostasis [8, 10]. ROS are inherent to cellular functioning and are present at low and stationary levels in normal cells. In vegetables, ROS are involved in metabolic processes related to photo protection and tolerance to different types of stress [13]. However, ROS can cause irreversible damage to DNA as they oxidize and modify some cellular components. Thus, ROS have a dual role, whether they will act as harmful, protective or signaling

factors, depends on the balance between ROS production and disposal at the right time and place [10]. In other words, oxygen toxicity can arise both from uncontrolled production and from the inefficient elimination of ROS by the antioxidant system.

Table 1.

The percentage of living cells in samples of RBCs before and after the H_2O_2 exposure.
The number of dead cells varies between 0-3 in all studied samples.

Group	Percentage of living RBCs
Intact RBCs	100 ± 0.0
RBCs + H_2O_2	99.94 ± 0.036

The results of the quantitative analysis of the carboxy-DCFDA fluorescence intensities revealed that the fluorescence intensity of carboxy-DCFDA is reverse comparative with the cell count in RBCs. With the decrease of fluorescence intensity of carboxy-DCFDA the cell count in RBCs increased up to $8*10^6$ (fig. 3). Thereby, the enhancement of fluorescence intensity leads to the magnification of cells' damage, and, consequently, decreases the number of cells. Vice-versa, the attenuation of fluorescence allows minimization the damage of cells and keeping the normal quantity of intact cells.

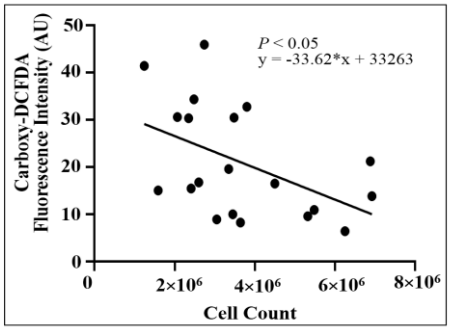

Fig. 3. Correlation analysis of two-photon fluorescent intensity in human RBCs vs. cell count *(number of participants).*

The method of two-photon microscopy imaging has a great significance for evaluation of oxidative stress in recent years. A group of researchers studied the plasma membrane fluidity alterations of macrophages caused by oxidative stress using Laurdan fluorescent dye [5]. Another interesting research is devoted to study the oxidative stress-mediated cell death in *Candida albicans* by two-photon microscopy [20]. Interestingly, cellular oxidative stress was studied by two-photon microscopy imaging using not only fluorescent dyes, but also pro-fluorescent nitroxides [16]. The method of two-photon microscopy was also used for the imaging of fluorescence lifetime of retinal pigment epithelial cells under normal and oxidative stress conditions [23].

Nevertheless, there is still a necessity to design effective and precise methods for detection of oxidative stress both in RBCs and living cells. In this study, we have concentrated our efforts on RBCs as they are unique mobile free radical scavengers owing to their ability to provide antioxidant protection not only to themselves but also all the cells in the organism by supplying with oxygen [30]. Besides, RBCs are not widely used in the studies, as it is very difficult to extract them and keep in native condition. Thereby**,** enhanced oxidative stress can be imaged in living RBCs by two-photon laser scanning microscopy making this a potentially useful and reliable approach to follow

oxidative stress lengthwise in the human living RBCs. Typically, the intentional, prolonged exposure to 1030 nm excitation pulses for imaging over 15 minutes did not induce measurable photo bleaching or photo damage to the RBCs.

The application of this novel approach can be limited by the need of using RBCs freshly isolated from blood samples. Moreover, it is important to note that the RBCs' antioxidant system is specific for each organism and varies from population to population.

Conclusion. In conclusion we proposed a novel approach for the qualitative and quantitative estimation of oxidative stress in human living RBCs that could successfully be applied in clinical research and testing of antioxidant compounds. Besides, it was shown that two-photon laser scanning imaging is a valuable tool for studying oxidative stress in living RBCs under oxidative stress related different pathological states.

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