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CORRELATION ANALYSIS BETWEEN OXIDATIVE STRESS IN LIVING ERYTHROCYTES AND HUMAN AGING BY TWO-PHOTON MICROSCOPY

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Human aging is the accelerating lesion of tissue and organ functions during a time. Simultaneously, progressive and irreversible accumulation of oxidative damage can be caused by reactive oxygen species (ROS). ROS are produced by various exogenous and endogenous factors, and their negative effects are neutralized by antioxidant defenses. The imbalance between ROS production and antioxidant defense mechanisms leads to oxidative stress which, in its turn, causes different age-related diseases. The goal of this study was to find and analyze the correlation between oxidative stress in red blood cells (RBCs) and human aging. For this purpose, two-photon laser scanning microscopy technique was applied. For determination of the superoxide dismutase, catalase, as well as ceruloplasmin ferroxidase activities, spectrophotometric analyze was carried out. Three main human aging groups were involved –young, middle and aged. The results showed that the two-photon fluorescence of carboxy-DCFDA, which specifies the oxidative stress intensity, was notably increased in RBCs with the increase of age ($p < 0.05$), and these intensities are in statistically essential positive correlation with age ($p < 0.001$) and a strong negative correlation ($p < 0.05$) with the catalase activity in RBCs and ceruloplasmin ferroxidase activity in plasma. Thereby, the analysis of oxidative stress in human living RBCs by two-photon microscopy can be proposed as a modern and brilliant tool for definition of human aging.

Human aging – reactive oxygen species – oxidative stress – human living erythrocytes – two-photon microscopy

Մարդու ծերացումը հյուսվածքների և օրգանների գործառույթների՝ ժամանակի հետ ա-րագացող խախտումն է: Միաժամանակ, օքսիդատիվ վնասվածքի զարգացող և անդարձելի կու-տակումը կարող է հարուցվել թթվածնի ակտիվ ձևերով (ԹԱՁ): ԹԱՁ-ն արտադրվում են զանազան էկզոգեն և էնդոգեն գործոններով, իսկ նրանց բացասական ազդեցությունները չեզոքացվում են հակաօքսիդանտային պաշտպանությամբ: ԹԱՁ-ի արտադրության և հակաօքսիդանտային պաշտ-պանության միջև անհավասարակշռությունը հանգեցնում է օքսիդատիվ սթրեսի, որն էլ իր հերթին հարուցում է զանազան տարիքային հիվանդություններ: Տվյալ հետազոտության նպատակն էր գտնել և վերլուծել արյան կարմիր բջիջներում (ԱԿԲ) օքսիդատիվ սթրեսի և մարդու ծերացման միջև հարաբերակցությունը: Այդ նպատակով կիրառվել է երկֆոտոն լազերային սկանավորող մանրադիտակման մեթոդը: Սուպերօքսիդ դիսմուտազի, կատալազի, ինչպես նաև ցերուլոպլազմին ֆերօքսիդազի ակտիվության որոշման համար իրականացվել է սպեկտրալուսաչափական վեր-լուծություն: Ներառված են եղել մարդկանց երեք հիմնական տարիքային խմբեր՝ երիտասարդ, միջահասակ և ծեր: Արդյունքները ցույց տվեցին, որ կարբօքսի-DCFDA-ի երկֆոտոն լուսարձա-կումը, որը բնութագրում է օքսիդատիվ սթրեսի ուժգնությունը, նկատելիորեն ավելանում էր էրիթրոցիտներում տարիքի հետ ($p < 0,05$), և այդ ուժգնությունները տարիքի հետ վիճակագրական

զգալի դրական հարաբերակցության մեջ են ($p < 0,001$), ուժգին բացասական հարաբերակցության մեջ են ԱԿԲ-ում կատալազի ակտիվության հետ և պլազմայում ցերուլոպլազմին ֆերօքսիդազի ակտիվության հետ ($p < 0,05$): Այսպիսով, մարդու կենդանի էրիթրոցիտներում օքսիդատիվ սթրեսի ուսումնասիրությունը երկֆոտոն մանրադիտակման միջոցով կարող է առաջարկվել որպես ժամանակակից և հրաշալի գործիք մարդու ծերացման որոշման համար:

Մարդու ծերացում – թթվածնի ակտիվ ձևեր – օքսիդատիվ սթրես – մարդու կենդանի էրիթրոցիտներ – երկֆոտոն մանրադիտակում

Старение человека – это ускоряющееся во времени нарушение функций тканей и органов. Одновременно прогрессирующее и необратимое накопление окислительного повреждения может быть вызвано активными формами кислорода (АФК). АФК продуцируются различными экзогенными и эндогенными факторами, а их негативные эффекты нейтрализуются антиоксидантной защитой. Дисбаланс между продукцией АФК и механизмами антиоксидантной защиты приводит к окислительному стрессу, который, в свою очередь, вызывает различные возрастные заболевания. Целью данного исследования было найти и проанализировать корреляцию между окислительным стрессом в красных кровяных тельцах (ККТ) и старением человека. Для этой цели был применен метод двухфотонной лазерной сканирующей микроскопии. Для определения активности супероксиддисмутазы, каталазы, а также церулоплазминфероксидазы был проведен спектрофотометрический анализ. Были задействованы три основные возрастные группы людей – молодой, средний и пожилой. Результаты показали, что двухфотонная флуоресценция карбокси-DCFDA, характеризующая интенсивность окислительного стресса, заметно увеличивалась в эритроцитах с возрастом ($p < 0,05$), причем эти интенсивности находятся в статистически значимой положительной корреляции с возрастом ($p < 0,001$) и сильной отрицательной корреляции ($p < 0,05$) с активностью каталазы в ККТ и активностью церулоплазминфероксидазы в плазме. Таким образом, анализ окислительного стресса в живых эритроцитах человека с помощью двухфотонной микроскопии может быть предложен в качестве современного и блестящего инструмента для определения старения человека.

Старение человека – активные формы кислорода – окислительный стресс – живые эритроциты человека – двухфотонная микроскопия

Various alterations in genetic, molecular, cellular, organ and system levels, as well as increasing damages in physiological functions lead to many age-related diseases [13].

Reactive oxygen species (ROS) have a detrimental impact on molecular mechanisms in human aging causing progressive and irreversible accumulation of oxidative damage, serious modifications of the organism's physiological functions and, consequently, leading to the development of various aging-related pathological states which influence on the lifetime [13]. Oxidative stress is a result of the balance disturbance between free radicals and antioxidants in the organism. The increasing levels of ROS are very harmful and cause negative impacts on tissues, cell membranes, lipids, proteins, DNA and other important components of the organism [16].

Aging is a progressive functional decay of the organism, which causes the increasing sensibility to various age-related diseases and finally, the death of the organism [3]. The reasons of aging are indefinite. According to the current theories, these can be based on damage concept, whereby the accumulation of damage (such as DNA oxidation) may cause biological systems to fail, or to the programmed aging concept, whereby problems with the internal processes (epigenomic maintenance such as DNA methylation) may cause aging [7]. Programmed aging should not be confused with programmed cell death apoptosis). Moreover, (there can be other reasons, which

can hasten the rate of aging in organism's beings like obesity [8] and attenuated immune system. Aging, in its turn, can be a serious reason for many different diseases, such as Alzheimer's disease, cardiovascular diseases, anemia, dementia, osteoporosis, diabetes, etc [11]. It is important to take into account, that the physiological aging is not less significant than the chronological age. It is based on molecular and/or cellular mechanisms of age. Understanding the mechanisms of physiological aging will help to find effective ways to treat various pathological states.

Consequently, there is a necessity to design suitable approaches to investigate the aging mechanisms and risk of mortality, which will also help to prevent the progress of age-related upsets, prolong the lifetime and increase the life quality. Nowadays, there are different molecular and clinical methods for the definition of the appearance of phenotypic age which will help to understand whether the individual younger or older than physiological age on molecular level. These methods comprise the estimation of telomere length [9], DNA methylation [10], as well as the comparative analysis of the characteristics between the individual and general population.

In this study we performed a modern approach for analysis of oxidative stress in human living red blood cells using two-photon microscopy imaging technique and also showed the correlation between oxidative stress and human aging. Two-photon microscopy imaging is a modern and useful tool to evaluate oxidative stress in human living RBCs under pathological states caused by oxidative stress [22]. Two-photon excitation microscopy is a fluorescence imaging technique that allows imaging of living tissue up to a very high depth, up to about one millimeter. Being a special variant of the multiphoton fluorescence microscope, it uses red-shifted excitation light which can also excite fluorescent dyes. Using infrared light minimizes scattering in the tissue. Two-photon excitation can be a superior alternative to confocal microscopy due to its deeper tissue penetration, efficient light detection and reduced phototoxicity [5].

Among all the cells in the organism, RBCs are one of the first cells affected by being continuously exposed to exogenous and endogenous sources of ROS [17]. However, circulating RBCs provide powerful antioxidant capacity for the whole blood owing to enzymatic (superoxide dismutase (SOD) and catalase) and non-enzymatic antioxidants. It allows maintaining the oxidant/antioxidant balance in the organism [12]. Various pathological states can induce uncontrolled ROS generation and change the activity of antioxidant enzymes in RBCs [1]. These mentioned circumstances highlight the advantages of studying oxidative stress in RBCs.

Therefore, the goal of this study was to find and analyze the correlation between oxidative stress in RBCs and human aging using two-photon microscopy imaging which can be an excellent tool for assessment of human aging mechanisms.

Materials and methods.

2.1 Study population

Active, living independently and healthy volunteers without any serious diagnosis were enrolled in this study (tab. 1). The participants were classified into three age groups: young age group including participants aged 20-40 years, middle-aged group including participants aged 40-60 years and aged group including participants at more than 60 years [20].

Table 1. Demographic Characteristics of the Healthy Volunteers Enrolled in the Study

Group	Young age group	Middle-aged group	Aged group
Number of participants (n)	15	15	15
Age range (years)	20 - 40	40 - 60	60 >
Gender (Male/Female)	8/7	8/7	8/7

2.2 Blood collection and sample preparation

Venous blood samples (2 ml) of healthy volunteers were collected in EDTA-containing tubes to prevent coagulation [25]. RBCs were isolated from the fresh blood samples using isotonic phosphate-buffered saline (PBS; $NaH_2PO_4 \times 2\ H_2O$ 123 mmol/l, Na_2HPO_4 27 mmol/l, NaCl 123 mmol/l; pH 7.4) solution according to the method described elsewhere [22] and were used for two-photon microscopy imaging immediately to avoid any modifications in erythrocytes' morphology.

2.3 Generation of an in vitro model of oxidative stress

To have additional control groups imitating the aging process, the RBCs from all the three age groups were exposed to additional *in vitro* oxidative stress by their incubation in 0.3 % peroxide solution at 37^0C for 40 minutes, after which the samples were washed twice in PBS at 2000 g at 4°C for 5 minutes [22].

2.4 Two-photon laser scanning microscopy imaging of oxidative stress in RBCs

For the detection of oxidative stress inside living cells, all the samples were treated with a membrane-permeable 5(6)-carboxy-2', 7'-dichlorofluorescein diacetate (carboxy-DCFDA, Sigma-Aldrich Chemie GmbH, Germany) fluorescent dye [22]. Microscopic slides of living RBCs for two-photon microscopy imaging were prepared according to the standard procedure described earlier [22]. All measurements were made at room temperature (20-22^0C).

Two-photon imaging was performed using a diode-pumped Yb:KGW ultrafast oscillator ("t-pulse", Amplitude Systems, France) available at the AREAL facility attached to a 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. Two-channel system with green filter was used providing 70 nm of full width at half maximum, 525 nm of maximum transmission and 92% of average transmission. A photomultiplier with 185-900 nm band width (R6357; Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany) was used to detect the carboxy-DCFDA fluorescence. A final power of 300 mW was maintained at the sample. Images were obtained by x, y galvanometric scanner in standard (512 × 512 pixels; 3.05 fps frame rate) modes on 12 bits photomultiplier with pixel clock of 1000 ns [22]. The mentioned parameters were maintained similar all over the experiments.

2.5 Detection of ceruloplasminferroxidase activity

The ferroxidase activity of ceruloplasmin was determined colorimetrically using ammonium iron (II) sulfate (Mohr's salt; $(NH_4)_2\,Fe\,(SO_4)_2 \times 6H_2O$) as a substrate [6]. Due to its ferroxidase activity, the ceruloplasmin is able to oxidize the bivalent iron contained in Mohr's salt in the complex with o-phenanthroline to a trivalent form, iron (III) ammonium sulfate, with a characteristic orange colored chromagen. Briefly, 230 μL of 0.45 M acetate buffer, pH 5.8, and 50 μL of 367 μM was added to the 5 μL plasma samples, then 20 μL of 5 mg/mL o-phenanthroline was added after 10 min. incubation at 37^0C. The optical density was detected at 420 nm (A_{420}) using a microtiter plate reader (Stat Fax 3200, Awareness Technology Inc.). The concentration of the substrate at the end of the reaction, which is inversely proportional to $\mathbf{A_{420}}$, was calculated based on the standards. For the control, the plasma and Mohr's salt were replaced by distilled water. For the two standards the plasma was replaced by distilled water or by 25 mM EDTA, respectively. The first standard is zero (A_{420} = 0 U/L), which represents the total ferrous concentration and the initial concentration of the substrate in the reaction mixture. In case of the second standard, EDTA binds all the ferrum imitating the total substrate oxidation ($\mathbf{A_{420}}$ = 2400 U/L). The ferroxidase activity of ceruloplasmin was expressed in activity units per 1 μL of the product, iron (III) ammonium sulfate, per 1 L of plasma per 1 minute (μM/min/L) and calculated using the following equation: $FAC = (C_1\text{-}C_2)/t \times V_1/V_2$, where FAC is the ferroxidase activity of ceruloplasmin, C_1 is the concentration of the substrate (Fe^{2+}) at the beginning of the reaction (120 μM/L), C_2 is the concentration of the substrate at the end of the reaction, t is the incubation time (10 minutes), V_2 is the total volume of the reaction mixture (350 μL) and V_1 is the plasma volume (5 μL).

2.6 Determination of SOD activity

The activity of SOD in the hemolysates was evaluated colorimetrically by measuring the inhibition level of adrenaline auto-oxidation by SOD in alkaline conditions and in presence of superoxide radicals [18]. Thus, 200 μL of 0.2 M bicarbonate buffer, pH 10.65, and 10 μL of 5.46 mM adrenaline hydrochloride was added to 10 μL of erythrocyte hemolysate with the subsequent

incubation at 37^0 C f or 3 minutes. The optical density was detected at 347 nm, as A_{347} represents the maximum absorption of the intermediate product of adrenaline auto-oxidation. The inhibition level was calculated using the following equation: $[1-(A_{347sample}/A_{347control})] \times 100\%$. For the control, the erythrocyte hemolysate was replaced by distilled water. For the calibrators, the erythrocyte hemolysate was replaced by commercial SOD (Sigma-Aldrich) in concentrations of 300, 240, 120, 60, 30 and 15 U/mL. The SOD activity was calculated according to the calibration curve representing the dependence of the level of inhibition of adrenaline oxidation from the SOD activity, which was expressed in units per mL of hemolysate (U/mL), where one unit represents the enzyme amount necessary to catalyze the formation of 1 μM of product or to cleave 1 μM of substrate for 1 minute in the conditions optimal for the enzyme activity.

2.7 Determination of catalase activity

The activity of catalase in the erythrocyte hemolysates was evaluated colorimetrically by the decrease of the H_2O_2 content in the reaction mixture and in the presence of the enzyme. Briefly, 50 μL of 0.08 % H_2O_2 and 100 μL of 0.02 M ammonium orthomolybdate were added to the 50 μL erythrocyte hemolysate with the subsequent incubation at 37^0C for 10 minutes. The optical density was detected at 420 nm, which is the maximum absorption of the H_2O_2 – ammonium orthomolybdate complex. For the control, the erythrocyte hemolysate was replaced by distilled water. For the calibrators, the erythrocyte hemolysate was replaced by commercial catalase (Sigma-Aldrich) in concentrations of 50, 25, 10 and 5 U/mL. The catalase activity was calculated by the decrease in the H_2O_2 concentration according to the following equation: $[1-(A_{420sample}/A_{420control})] \times 100\%$. The catalase activity was calculated according to the calibration curve representing the dependence of the absorption (A_{420}) from the catalase activity, expressed in U/mL.

2.8 Image processing and statistical analysis

The images were processed using the Fiji/ImageJ software (ImageJ 1.50 i NIH, Bethesda, MD, USA) [15] as described previously [22]. The 0-255 normalized scale was used for the selected images, where 0 corresponds to the black and 255 corresponds to the white color. The image intensity was calculated as a sum of intensities of all the cells from the ROI. The following formula was used to calculate the corrected total cell fluorescence (CTCF). CTCF = Integrated density - (Area of selected cell x Mean fluorescence of background readings).

Statistical analysis was performed using "Graphpad Prism 8.0.1" (GraphPad Software Inc., USA) and one-way or two-way ANOVA followed by Tukey's post-tests. Data is presented in bar graphs showing mean ± SEM (standard error of the mean). The degree of linear regression was assessed for correlation analysis between the studied parameters using Pearson correlation coefficient. Subsequently, Pearson's r and *P* values were calculated to evaluate the effects of any difference and the correlation equations were developed. *P*-values less than 0.05 were considered statistically significant.

Results and Discussion.

3.1 Imaging of human living erythrocytes by two-photon microscopy

Human living erythrocytes of three age groups (young, middle aged and aged) were imaged by two-photon laser scanning microscopy, and the results of these experiments were presented in figures 1 and 2. It was shown that oxidative stress was increasing simultaneously with the increasing of the age. In accordance with the results, the Carboxy-DCFDA fluorescence mean intensities increased 3.8 ($p < 0.0001$) and 1.9 ($p < 0.05$) times in aged group in comparison with the young and middle aged groups, respectively.

The same tendency was observed in the aging modeling where H_2O_2 was used for the imitation of the aging process. In imitated pathological aged (aged RBCs+H_2O_2) and imitated middle aged group (middle aged RBCs+H_2O_2) there was detected 2.4 ($p < 0.01$) and 2 ($p < 0.05$) times statistically significant increase in comparison with the young ("young RBCs+H_2O_2") group, respectively.

Nevertheless, no any statistically essential differences were observed between the imitated pathological aged (aged RBCs+H_2O_2) and imitated middle aged groups (middle aged RBCs+H_2O_2).

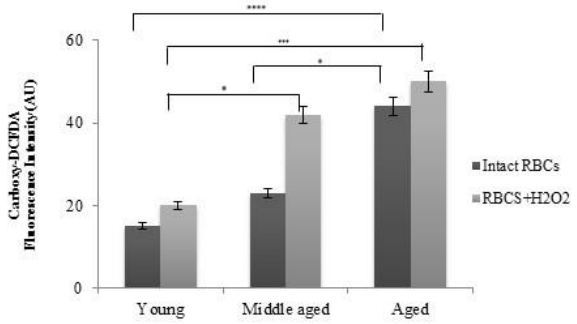

Fig.1. Carboxy-DCFDA fluorescence mean intensities in three age groups, young, middle aged and aged. *Fluorescent intensities are represented in arbitrary units (AU; mean ± SEM).*
**p < 0.05; ***p < 0.001; ****p < 0.0001.*

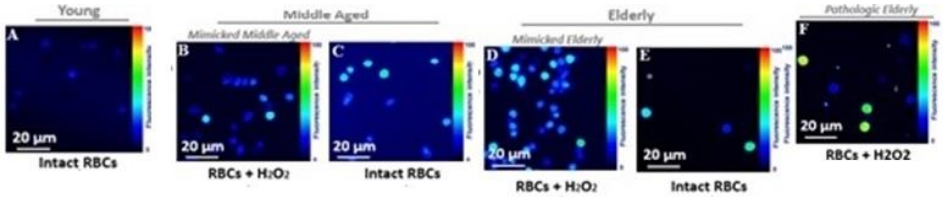

Fig.2. Representative two-photon fluorescence intensity images of RBCs from young, middle aged and elderly people treated (B, D, F) and non-treated (intact) with H_2O_2 (A, C, E).

3.2 Estimation of antioxidant system

The results of the activities of superoxide dismutase and catalase, as well as the ceruloplasminferroxidase activity are presented in fig. 3. The activity of superoxide dismutase was decreased with the increasing of the age. What concerns the catalase activity, a small, but essential 1.2 times ($p < 0.01$) decrease was detected in blood plasma of aged people in comparison with the young people. Vice versa, the results were highly substantial in case of evaluation of ceruloplasminferroxidase activity. Small, but statistically significant ($p < 0.05$ and $p < 0.0001$, respectively) reduction of ceruloplasminferroxidase activity were detected in the blood plasma of middle aged and aged people in comparison with the young people, and the difference between the middle aged and aged people was statistically essential ($p < 0.0001$).

3.3 Age-dependent analysis of two-photon fluorescent intensity in RBCs and its correlation with antioxidant enzymes activity

A strong, statistically substantial positive correlation was found between the two-photon fluorescent intensities of oxidative stress in RBCs and age ($p < 0.001$), as well as a strong, statistically substantial negative correlation ($p < 0.05$) was found between the two-photon fluorescent intensities of oxidative stress in RBCs and both activity of catalase and ceruloplasminferroxidase activity in plasma (fig. 4).

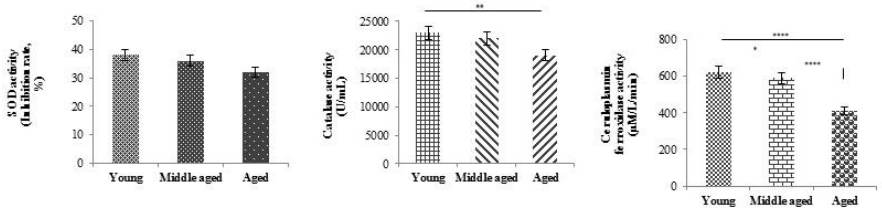

Fig.3. The activities of superoxide dismutase (SOD), catalase, as well as the ferroxidase activity of ceruloplasmin in the blood plasma of young, middle aged and elderly people (M ± SEM**)**. **p < 0.05; **p < 0.01; ****p < 0.0001 (number of participants, n = 15 in each group)*.

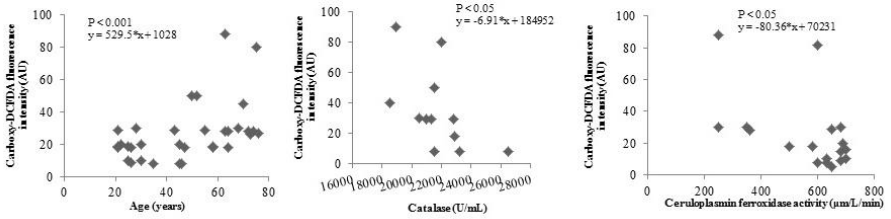

Fig.4. Correlation analysis of two-photon fluorescent intensity in human RBCs vs. age, activity of catalase and ferroxidase activity of ceruloplasmin *(number of participants, n = 15 in each group)*.

Aging, which can be classified as pathological and physiological aging, is a complex biological process characterized by functional decline of tissues and organs, structural degeneracy, reduced adaptability and resistance, all of which contribute to an increase in morbidity and mortality caused by multiple chronic diseases [2]. With its progress, the aging increases one's sensibility to diseases associated with this process [11]. Aging-related diseases perform a serious danger to human health and reduce the quality of life among aged people. Furthermore, it has become a global difficulty to clarify the mechanisms of aging, retard the process of aging, decrease the occurrence of age-related diseases, and keep the fadeless appearance during the aging process.

In this study we suggested a modern and proved approach for the correlation analysis between oxidative stress in living RBCs and human aging using two-photon laser scanning imaging technique which exactly illustrates the aging processes accurately showing the increase of oxidative stress with the increase of the age. For this purpose, 6 main groups were examined for two-photon microscopy imaging of oxidative stress in human aging: young intact RBCs, young RBCs with H_2O_2, middle age intact RBCs, middle age RBCs with H_2O_2, as well as aged intact RBCs and aged RBCs with H_2O_2.

The additional H_2O_2 induces quite high variations in the intensities in young people, while in aged people the changes are very low showing an interesting tendency of reaching the saturation level when additional H_2O_2 doesn't cause more oxidative stress but already leads to death conditions.

Antioxidant system plays a crucial role for elimination of free radicals. This system includes significant enzymes for antioxidant defense, such as catalase, superoxide dismutase and glutathione peroxidase. Cells contain a large number of antioxidants to prevent or repair the damage caused by reactive oxygen species, as well

as to regulate redox-sensitive signaling pathways. The SODs convert superoxide radical into hydrogen peroxide and molecular oxygen, whereas the catalase and peroxidases convert hydrogen peroxide into water. In this way, two toxic species, superoxide radical and hydrogen peroxide are converted to the harmless product water [24].

That is why, it was important to study the activities of these antioxidant enzymes and find the correlation of these activities with human aging. Two-photon laser scanning imaging technique allows detection statistically essential alterations even in small groups, and this fact one more time highlights and confirms the great value of this approach for evaluation of physiological aging. Furthermore, we showed that the two-photon fluorescence intensities reflecting the oxidative stress in human RBCs not only positively correlate with the age, but also negatively correlate with the activity of catalase and ceruloplasmin, one more time confirming the propriety and sensitivity of this method.

Interesting results were performed by another group of researchers which studied antioxidant enzymes in the aging human retinal pigment epithelium [14]. They investigated the effects of aging and macular degeneration on retinal pigment epithelium catalase or superoxide dismutase activities. According to the results of these investigations, superoxide dismutase activity showed no significant correlations with aging or macular degeneration, while catalase activity decreased with age ($p < 0.02$) and macular degeneration ($p < 0.05$) in both macular and peripheral retinal pigment epithelium [14]. The changes in activities of catalase and superoxide dismutase associated with age were studied in the rat brain [23]. The results showed that the total SOD and Mn SOD activities increased with age and exhibited higher levels at 6 and 12 months but decreased thereafter. Activity of catalase showed a similar tendency and especially peaked at 12 months [23].

Although the investigation of oxidative stress in living erythrocytes and its correlation with human aging is very interesting and prospective owing to specificity of these cells, but it is not less interesting to analyze the age-dependent and oxidative stress-mediated changes in the expression of catalase and superoxide dismutase in human granulosa cells (GCs) [21]. It was shown that the expression of SOD1, SOD2 and catalase was decreased at the protein level in women ≥38 years ($p < 0.05$). Examination at an ultrastructural level revealed that most of the GCs from this group showed defective mitochondria and fewer lipid droplets than those observed in the younger group. Consequently, GCs from older patients suffer from age-dependent oxidative stress injury and are taken as an evidence for reduced defense against reactive oxygen species (ROS) in GCs during reproductive aging [21].

Aging measures can be divided into 2 types, the population-level and the individual-level [4]. There are various measures of population aging, such as chronological, functional, economic, etc [19]. The individual aging is more complicated as there are also the individual's physiological and biological declines that should be analyzed. For this purpose, it is very important to find and assess the molecular and cellular biomarkers of aging.

Conclusion Finally, two-photon laser scanning imaging technique is a brilliant tool for analysis and estimation of the oxidative stress in human living erythrocytes, as well as for detection of its correlation with human aging which will allow to deeply understand the mechanisms of human aging, develop appropriate strategies for slowing down the aging processes and increase the quality of life.

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