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Noninvasive Method of Determining Skin Antioxidant/Oxidant Activity: Clinical and Cosmetics Applications

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Abstract- Free radical oxidation ensures regular metabolism in human body. In case of oxidant/antioxidant misbalance free radical oxidation may lead to oxidation of lipids, membrane destruction and irreversible changes in DNA. Many diseases are both causes and consequences of oxidative stress. Its monitoring is essential for selecting and using modern therapy, thus reducing and preventing oxidative stress and irreversible changes in human body. The article describes a new electrochemical method of determining antioxidant/oxidant activity (AOA/OA) of skin. The source of information is the platinum electrode potential shift observed when the investigated part of skin came into contact with a medium containing mediator system. An ECG-type electrode served as a reference one.To prove the reliability of the data obtained with the proposed method, model systems, containing antioxidants or oxidants in different concentrations were investigated. The data obtained showed high self-descriptiveness and reliability of the results. The data demonstrating the impact of cosmetic creams on skin AOA are presented. The interrelation between skin AOA/OA and the presence of cardiovascular pathologies is proved.

Keywords- Potentiometry, Oxidant Activity, Antioxidant Activity, Skin, Noninvasive, Cosmetics, Cardiovascular Pathologies

1. INTRODUCTION

There are strong links between oxidative stress (OS) and human body disorders. OS is believed to contribute to the development of a wide range of diseases including Alzheimer's disease, Parkinson's disease, diabetes, rheumatoid arthritis, neurodegeneration, inflammation, cardiovascular, and aging. OS is caused by excessive synthesis of Reactive Oxygen Species (ROS) that is followed by oxidation chain reactions. The ROS excess is accompanied by peroxides and synthesis of peroxides transformation products [1], cell membrane [2] and DNA [3] damage .

In many cases, the starting factor is not clear: whether oxidants cause disease or ROS are produced as a consequence of disease. Two types of factors leading to OS should be taken into account: endogenous and exogenous [4]. Actually, solar energy and other types of irradiation, environment pollution seems to be endogenous factors, which are, obviously, the most important for skin [5]. Thus the conclusion might be drawn that OS is the cause of disease. As for inner pathologies it might be assumed that the primary factor is a disease itself, however special studies need to be taken to prove this assumption .

Skin is the first line of defense from endogenous oxidative attacks. On the other hand, a variety of systemic and internal pathological conditions may be reflected in skin. These include diabetes mellitus [6], artherosclerosis [7-8], inflammatory bowel diseases (IBD) [9], mental stress [10], and aging [11]. Moreover, OS may contribute to adverse effects on skin, such as erythema, edema, wrinkling, inflammation, autoimmune manifestation, hypersensitivity, keratinization abnormalities, preneoplastic lesions and skin cancer [12].

Living organisms have developed complex and integrated extracellular and intracellular defense systems against stress caused by reactive oxygen and nitrogen species (ROS, RNS). Enzymatic and non-enzymatic antioxidants are the principal components of the defense system. Enzymes are exogenous antioxidants. However we will leave enzymatic part of antioxidants beyond this article and consider non-enzymatic endogenous/exogenous antioxidants as their level can be more or less easily monitored and regulated by therapy and food .

Two groups of monitoring methods are worth paying attention to. The first group includes methods of measuring antioxidant activity in food, nutrients, biological liquids [13-15] and tissues [16]. The second group allows detecting oxidation products [17] that are used for assessing body condition. However in most cases the methods of the second group provide indirect or post factum information only, when irreversible damage has already been caused. In practice the methods of body condition evaluation are invasive.

As skin, on the one hand, is the first line of defense from external factors and, on the other hand, the object of endogenous impact, it is natural to assume that its oxidant/antioxidant activity is affected by these factors. Therefore, skin can serve as an indicator of endogenous and exogenous disorders. The latter can become a basis for non-invasive analytical assessment methods of AOA/OA.

The Raman spectroscopy method [16] can estimate the level of carotenoids. A drawback of this method is that it allows to determine the level of carotenoids only, while the pool of skin antioxidants includes fermentative antioxidants (superoxidedismutase, catalase, peroxidase, glutathione peroxidase and others) and low-molecular nonenzymatic antioxidants that are present both in cell membranes (tocopherols, ubiquinol, vitamin A, its precursor carotenotene) and in epidermal and dermal extra cellular tissue (thiol-containing compounds, ascorbic acid, urates). Thus, carotenoids are just one form of a variety of antioxidants in skin, and consequently their determination cannot provide sufficient information about antioxidant activity as the integral parameter. In addition, carotenoids possess the ability to quench $^1O_2$ only.

The work [18] can be mentioned here. Indirect evaluation of AOA is described in it. Authors use as source of information decrease of induced skin redness and blood flow. Moreover, it is difficult to accept the method described there as really non-invasive, because treatment of skin by methyl nicotinate is used to induce a circular erythema before measurements.

Taking into account the fact that the reactions leading to AOA/OA balance in human body are of electrochemical nature, the use of electrochemical methods for determining this parameter seems the most perspective. One of the first attempts to implement this method might have been the method described in [19], which included washing antioxidants from the surface of skin into the solution with subsequent cyclic voltammetry measurements. The real direct noninvasive method was described in Patent [20]. The method is based on bringing the studied object into contact with electroconductive solution containing $FeCl_3$, pH < 2, or an aqueous solution containing ADP-Fe(III), or I_2/NaI and consequent evaluation of AOA/OA by potential changes of the electrodes immersed in the electroconductive solution. The working electrode and reference electrode were immersed in a half cell filled with test solution, which at the last case allowed determining both AOA and OA.

In [21-23] the results of theoretical and experimental investigations on potentiometric method of skin AOA/OA measurement were presented.The potential change was measured for 10 minutes. The calculated AOA/OA provided direct information about the condition of the subject under investigation.

Advantages of the proposed approach are as follows: skin AOA/OA can be measured as integrated parameter by a direct, highly sensitive, user friendly and selective method.

The goals of this work are: (i) to describe experimental details of the new noninvasive method of investigating skin AOA/OA; (ii) to prove its reliability and self-descriptiveness; (iii) to demonstrate possibility of application of the method for investigating the impact of cosmetic creams on skin AOA; (iv) to give evidence for interrelation between skin AOA/OA and cardiovascular pathologies.

2. EXPERIMENTAL

2.1. Methodand calculations

Theinformation source with regard to OA/AOA is the electrode potential shift in the mediator system $K_3[Fe(CN)_6/K_4[Fe(CN)_6]$, which is observed when antioxidants or oxidants (the sample) are introduced into the medium. This shift results from changes in the concentration of mediator oxidized/reduced forms during the reaction (1) for antioxidants and reaction (2) for oxidants:

$$Fe(III) + AO = Fe(II) + AO_{Ox} \quad (1)$$

$$Fe(II) + OA = Fe(III) + OA_{Red} \quad (2)$$

Where AO – antioxidant; AO_{Ox} –antioxidant oxidation product; OA – oxidant; OA_{Red}– oxidant reduction product.

AOA and OA are expressed in mole-eq/l (M-eq) and calculated applying Equations 3and 4 given below [21-23]:

$$AOA = \frac{C_{Ox} - \alpha C_{Red}}{1+\alpha},\ \alpha = (C_{Ox} / C_{Red}) \cdot 10^{(E_1 - E)nF / 2.3RT} \quad (1)$$

$$OA = \frac{\alpha C_{Red} - C_{Ox}}{1+\alpha},\ \alpha = (C_{Ox} / C_{Red}) \cdot 10^{(E_1 - E)nF / 2.3RT} \quad (2)$$

where E – initial potential, V; E_1 – end potential, V; C_{Ox} – concentration of oxidized form of mediator system, M; C_{Red} – concentration of reduced form of mediator system, M; AOA – antioxidant activity, M-eq; OA–oxidant activity, M-eq. In this case expressions of concentration in M and M-eq are agreed as one electron participates in electrochemical reaction.

Accuracy and reliability of results obtained with this method were confirmed by comparing them with the results obtained with the use of TAS Randox method [14].

2.2. Instruments and electrodes

In order to investigate cream AOA the multifunctional potentiometric analyzer MPA-1 (IVA, Russia), platinum screen-printed working electrode (IVA, Russia) electrode and

silver/silver chloride reference electrode (Gomel Plant of Measuring Devices, Republic of Belarus) were used.

For potentiometric skin and model systems investigations the pilot sample of potentiometric analyzer AOT-1 (IVA, Russia) was used as an interface to a PC. A platinum screen-printed electrode (IVA, Russia) was used as a working electrode. An ECG-type electrode (Arbo, «Kendall», USA) served as a reference electrode. The design of a unit for measuring skin AOA/OA is shown in Fig. 1 [21-22].

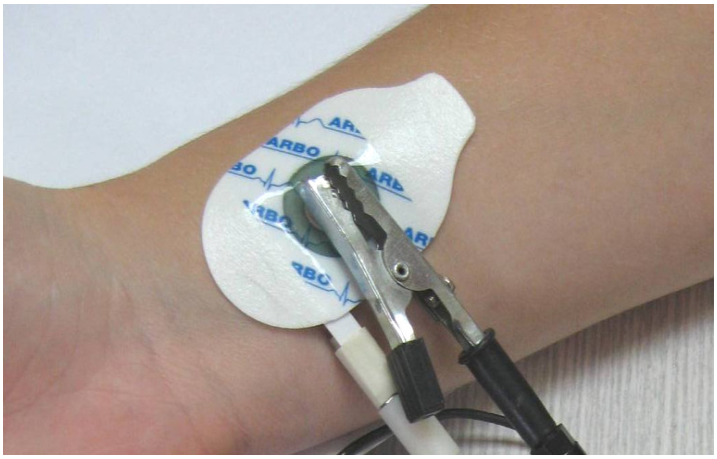

Fig. 1. Unit for measuring skin AOA/OA

Ultrasonic Processor VCX 750, ultrasonic disperser (Sonics & Materials, Inc, USA) was used to emulsify creams.

2.3. Materials

$K_4[Fe(CN)_6]$, $K_3[Fe(CN)_6]$, KH_2PO_4, $Na_2HPO_4.12H_2O$ (Reachim, Russia) chemically pure; highly conductive gel (Geltek-Medica, Russia) was used to prepare mixture of gel and mediator system. Uric acid, ascorbic acid, glutathione (Sigma, USA), and hydrogen peroxide (Reachim, Russia) were used as model objects. Precise concentration of hydrogen peroxide was achieved with titration by potassium permanganate. Gel containing, mediator system and antioxidant or oxidant was taken as model systems.

Creams were bought in the stores. They were produced by: *Silky Hands*, hand cream (LLC Concern Kalina, Russia); cream-balm for hands (LLC First Solution, Russia); *2-in-1 Protecting Hand & Nail Cream* (Oriflame Cosmetics, Sweden); *Solutions. Energy minerals. 30+,* anti-aging face day creams (AVON Products Inc., USA); *Caprute Sculpt 10 Nuit*, regenerating firming night cream (Christian Dior, France); *Cream to strengthen and repair*

the skin Anti-age, body cream (Natura Siberica Cosmetics, Russia); *Black Pearl Skin. Program 56+*, face day creams (LLC Concern Kalina, Russia).

2.4. Investigated subjects and measurements modes

2.4.1. Electrodes

The pair of investigated electrodes (Pt- ECG-type or ECG-type - ECG-type electrodes) was immersed in gel containing the mediator system ($K_3[Fe(CN)_6/K_4[Fe(CN)_6]$). The mixture was applied to Teflon film. Potential difference was measured between these electrodes over time.

2.4.2. Model systems

Model systems were prepared mixing conductive gel, mediator system (10^{-3} M $K_3[Fe(CN)_6]+10^{-5}$ M $K_4[Fe(CN)_6]$ in gel) and one of antioxidants or oxidants. The mixture is applied on Teflon film. A pair of electrodes (Pt-ECG-type electrode) was immersed into the mixture. Potential difference was measured.

2.4.3. Cosmetic cream

0.01 g of cream was mixed with 5 mL of water. Mixture was emulsified in ultrasonic field at 40 kHz for 2 min. Water emulsions of the studied creams were stable for the whole measuring period due to large content of surfactant species in it Emulsion was placed into electrochemical cell, containing mediator system 10^{-3} M $K_3[Fe(CN)_6]+5\times10^{-5}$ M $K_4[Fe(CN)_6]$ in buffer solution and two electrodes: Pt as working one and silver/silver chloride reference electrode. Potential difference was measured.

2.4.5. Skin

The surface of human skin in the wrist area served as the subject of investigation. The investigated area of skin was washed with deionized water. After cream application and before measurement, excessive amount of cream was removed with sterile tissue.

Conductive gel containing 10^{-3} M $K_3[Fe(CN)_6]+10^{-5}$M $K_4[Fe(CN)_6]$ was applied to skin, the measuring electrode (Pt) and reference (ECG-type) electrode were placed on the surface of gel. The change in the potential of the system was recorded over time.

3. RESULTS AND DISCUSSION

3.1. Electrodes investigation

Potential differences between a number of pairs of electrodes (Pt-ECG) and pre-logarithmic coefficients in the Equation $E=E^0+b\cdot\lg(C_{Ox}/C_{Red})$, which were observed in mediator systems $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ differing by components concentration introduced into conductive gel are given in Table 1.

Table 1. Potential differences between pairs of electrodes (Pt-ECG) and pre-logarithmic coefficients in the Equation $E=E^0+b\log(C_{Ox}/C_{Red})$ observed at different concentrations of the mediator system $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ introduced into conductive gel

C_{Ox}/C_{Red}, M/M	ΔE, mV			
	Pair 1	Pair 2	Pair 3	Pair 4
0.001/ 0.0001	–	–	239	234
0.0001/ 0.000005	235	246	256	252
0.001/ 0.00001	275	285	–	–
d(ΔE)/ d(lg (C_{Ox}/C_{Red}))	57	56	57	60

Potential difference between pairs of electrodes Pt-ECG and the pair of two -ECG electrodes are given in Fig. 2.

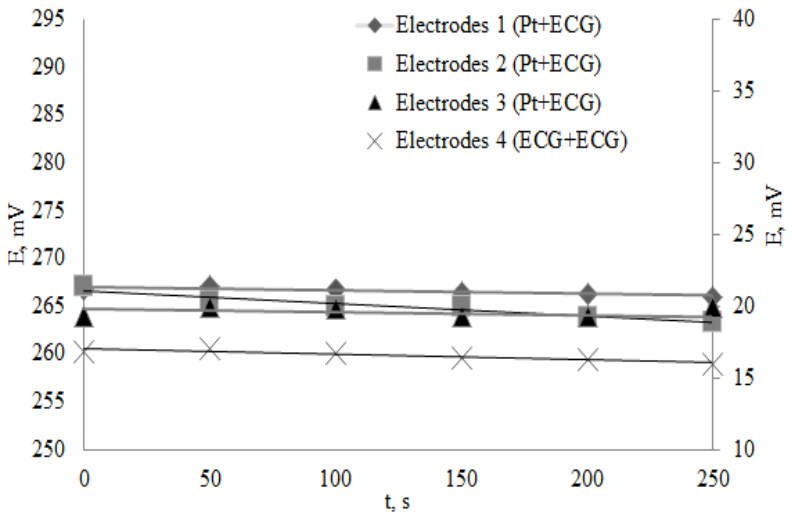

Fig. 2. Potential difference between pairs of electrodes 1-3 (Pt-ECG) are given on the left axis and potential difference between two-ECG electrodes (4) is given on the right axis

It is apparent from Table 1 and Fig. 1 that the uses of different pairs of electrodes (Pt-ECG-type) do not provide the one and the same potential. The reason is that ECG electrodes are not identical; it is seen from the curve given on the Fig. 1. Potential difference between

two at random chosen electrodes is about 12 mV. But the important factor here is the stability of the potential of the electrodes in time. In addition it is necessary to take into account that the information source is potential difference between the initial and finite values of the same pair of the electrodes. Calculation of the AOA/OA (concentrations) are reliable so far as in all cases pre-logarithmic coefficient in the equation E=f(log(C_{Ox}/C_{Red})) is close to the Nernstian one. This data confirm the correct choice of electrodes (Pt and ECG) for measurements.

3.2. The study of model solutions of antioxidants and oxidant

The findings of the study are presented in Table 2.

Table 2. Correlation between introduced and found concentrations of antioxidants or oxidants in model systems

Antioxidant or oxidant	Introduced, 10^5 M-eq	Found, 10^5 M-eq	S_r	Recovery, %
Ascorbic acid	2	1.88±0.41	0.08	94
	4	4.04±0.24	0.02	101
Glutathione	2	1.77±0.30	0.06	89
	4	4.17±0.30	0.03	104
Uric acid	2	1.97±0.28	0.06	99
	4	4.04±0.24	0.02	101
Hydrogen peroxide	1	0.86±0.17	0.10	86

The data in Table 2 prove the following: (i) the results are accurate: the 'introduced' and 'found' values correlate; (ii) the results can be obtained with insignificant discrepancies in true values.

3.3. The study of accuracy and reproducibility of skin AOA/OA measurement

In order to study accuracy of measurement results we use the problem solution described in [22].

$$\vartheta(t) = \frac{c_0\sqrt{4Dt}}{\delta\sqrt{\pi}} \qquad (3)$$

The expression relates AOA/OA concentration in skin (c_0) to average concentration of the mediator system component ($\vartheta(t)$) resulting from Reactions (1) or (2) in gel. It is reasonable to suppose that accuracy of the results of the analysis is ensured by the fulfilling of the condition when $\vartheta(t)=c_0$. At the same time $\sqrt{4Dt}/\sqrt{\pi}=\delta$, where δ is the thickness of a diffusion

layer. Being aware of the fact that the described approach is idealized we suppose, however, that it can be used for estimations given below. Taking measurement time equal 10 min and the coefficient of AO diffusion close to the coefficient of glucose diffusion in skin as presented in [24], $D \approx (2.56 \pm 0.13)\times10^{-6}$ cm^2, we obtain $\delta\approx4\times10^{-2}$ cm with epidermis thickness (0.04 ÷ 0.09) cm. These results make us assume that under the chosen experimental conditions, $\vartheta(t)$ should be close to c_0 and the result could be quite accurate.

The effect of hard-to-account non-reproducibility of gel thickness between skin and electrode on an error of analysis is presented in Table 3. Skin AOA was measured three times on different inner areas of a right arm.

Table 3. Reproducibility of results of skin AOA measurement

No.	AOA, 10^5 M	X ± ΔX	S_r
Experiment 1	1.15	1.16 ± 0.15	5.21
	1.22		
	1.10		
Experiment 2	2.04	2.04 ± 0.31	6.11
	2.17		
	1.92		

Thus, the chosen conditions of the experiment ensure accurate and reproducible results.

3.4. Cosmetic investigations

Antioxidants are one of the major ways to slow the impact of free-radical damage. It is difficult to overestimate their importance for human body (and skin as the body's largest organ). A great majority of creams is directed to reducing age effect on skin that is partly connected to AO action. So, AO is useful to be monitored. Above described new investigation method can give valuable direct information on antioxidants impact in skin.

Different creams were investigated here. The results of its comparison are presented on Fig. 3. It is seen, that *Black Pearl Skin Program 56+* face day cream contains the largest amount of antioxidants.

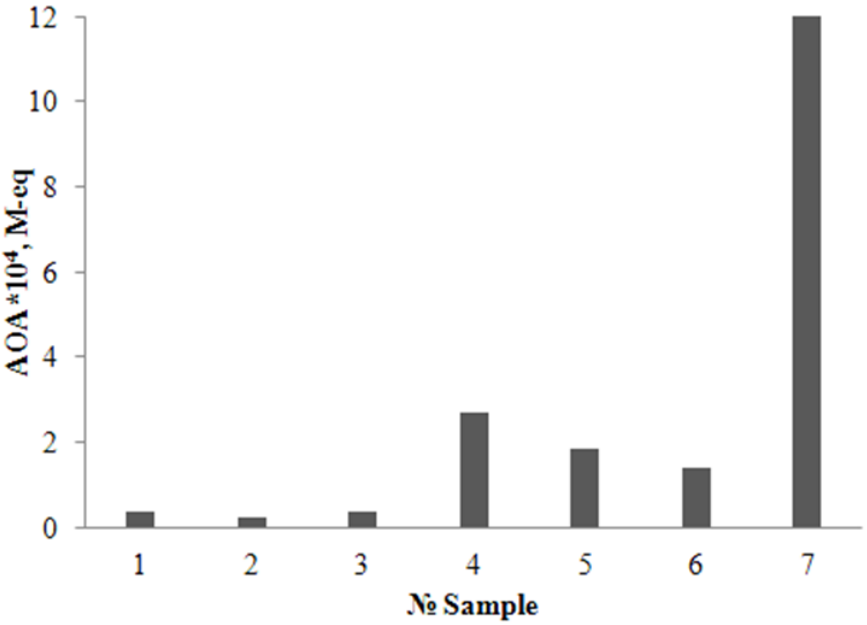

Fig. 3. AOA of cosmetic creams: 1) Silky Hands, hand cream (LLC Concern Kalina, Russia); 2) Cream-balm for hands (LLC First Solution, Russia); 3) 2-in-1 Protecting Hand & Nail Cream (Oriflame Cosmetics, Sweden); 4) Solutions. Energy minerals 30+, anti-aging face day creams (AVON Products Inc., USA); 5) Caprute Sculpt 10 Nuit, regenerating firming night cream (Christian Dior, France); 6)Cream to strengthen and repair the skin Anti-age, body cream (NaturaSiberica Cosmetics, Russia); 7)Black Pearl Skin. Program 56+, face day creams (LLC Concern Kalina, Russia)

Most creams directly target to reduce age effect on skin. The resultsof *Silky Hands* cream and *Black Pearl Skin Program +56* cream applications on skin are compared in Figs. 4 and 5. Fig.4 illustrates the impact of *Silky Hands* cream on 5 volunteers' skin. Skin AOA was measured before cream application, 20 min, 8 h and 24 h after cream application. An increase in the skin AOA for all members of the study group was first observed 20 min after cream application. After 8 h the skin AOA of four members of the group was higher than before cream application, moreover the skin of three persons demonstrated an increase in this parameter over time. After 24 h of cream application skin AOA started to decrease but three persons had higher AOA than before cream application. It is worth mentioning that the effect was more noticeable for the group members aged 40+.

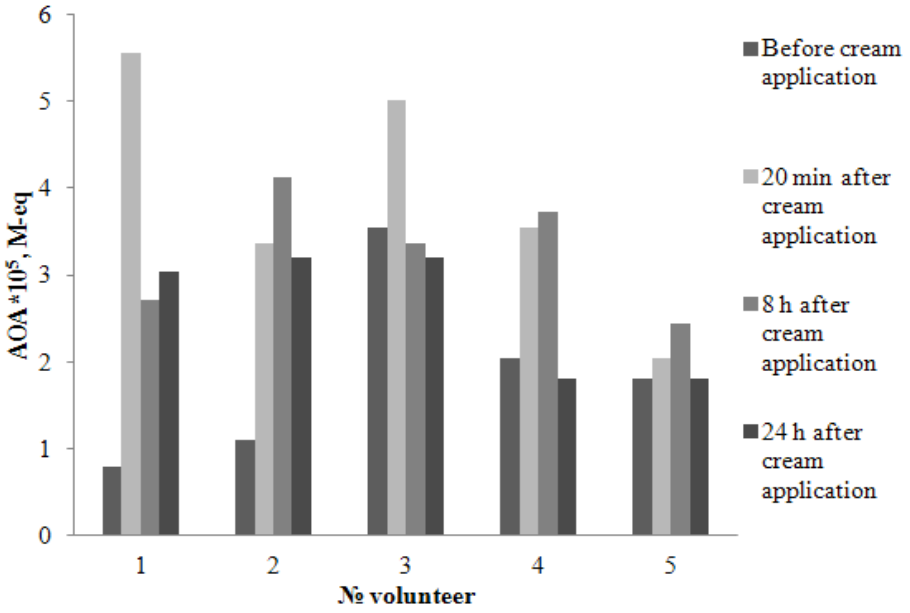

Fig. 4. Skin AOA before and after *Silky Hands* cream application

The impact of *Black Pearl Skin Program 56+* was studiedfor two age groups: (1) volunteers aged 22-27 (Fig. 5a) and (2) volunteers aged 45+ (Fig. 5b). Measurements were taken before cream application and 30 min after cream application. Fig. 5 shows that the effect of the cream on the skin of volunteers age group (2) is more pronounced (Fig. 5b) than the effect of the cream on the skin of young volunteers (Fig. 5a) Theresults are expected as the cream is intended for this particular age group and is rich in antioxidants (Fig.3) which prevent premature skin aging.

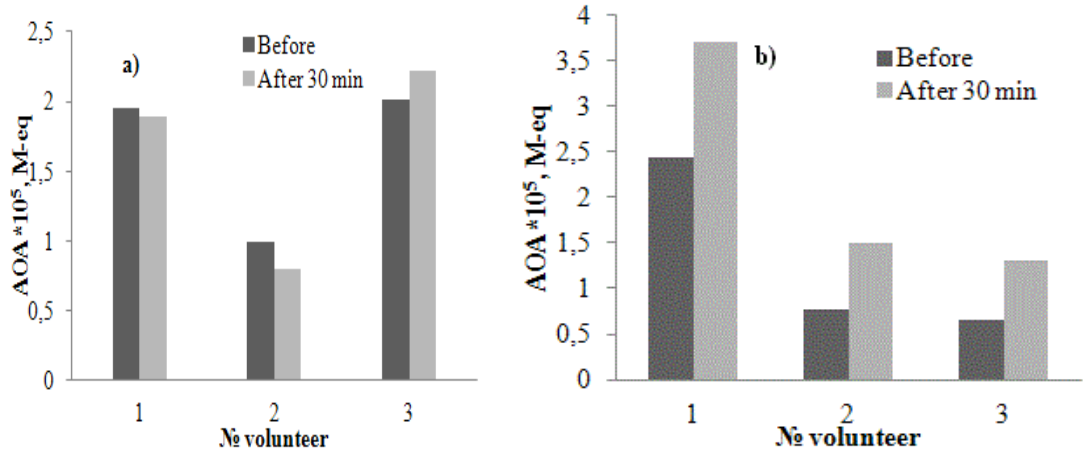

Fig. 5. Skin AOA before and after *Black Pearl Skin Program +56* cream application: a) age group 22-27; b) age group 45+

3.5. Clinical investigations

Oxidative stress plays an important role in pathogenesis of cardiovascular disorders, including arterial hypertension and its complications. As arterial hypertension is a disorder with wide abundance in population, the need for simple and safe test for oxidative stress diagnostic in group of hypertensive patients is obvious.

We assessed skin AOA/OA of patients with arterial hypertension and healthy people aged from 20 to 25 (Fig. 6) and compared the results with other clinical and laboratory data. Skin AOA/OA decreased in the group of hypertensive patients as compared with healthy people.

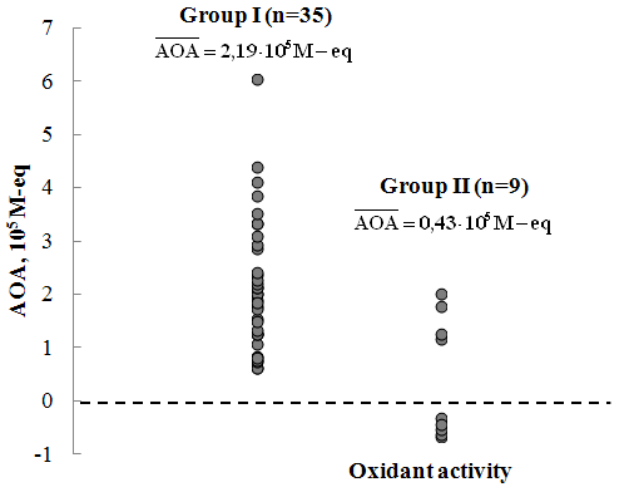

Fig. 6. Skin AOA/OA of patients with arterial hypertension (Group II) and healthy people (Group I)

Key findings of this investigation showed that the most significant decrease in skin AOA/OA was associated with:

- Poor compliance of patients (independently from duration of a disorder, hypertension was not controlled by medications in this group of patients). Those patients had OA instead of AOA (see lower part of Fig.6).
- Dyslipidemia.
- Tortuosity of large (carotid and vertebral) arteries of neck, obtained by duplex colored ultrasound.

On the other hand, good compliance of patients (continuous treatment of arterial hypertension) was accompanied by higher skin AOA/OA.

The combination of these factors for patients with lower skin AOA/OA possibly can serve as an indicator of severe oxidative stress in this group of people. It can be explained by the fact that non-treated hypertension causes the most serious hydrodynamic damage of vessels, which creates prolonged tension of an arterial wall, leading to its deformation (tortuosity).

The deformation of an arterial wall along with dyslipidemia leads to endothelial dysfunction, endothelial cells damage and oxidative stress strengthening.

A decrease in skin AOA/OA may mean severe oxidative stress and endothelial dysfunction in small vessels of skin, whose endothelial cells are also damaged by high blood pressure. As we have no aim to study correlation between the level of blood pressure and the level of AOA/OA, we did not establish the correlation between AOA/OA and the status (the stage) of arterial hypertension in this paper. More of all, in our work some patients, suffering from hypertension, had the same AOA/OA level, as the healthy people. But the hazard of poor compliance with the treatment of arterial hypertension is well known, and itsmorphological basis – the damage of the vessel wall, manifesting by oxidative stress, is well described. As we have obtained in hypertensive patients with poor compliance the OA instead the AOA, dyslipidemia, tortuosity of arteries of neck, we propose to use our method for the screening of hypertensive people as the possible tool for treatment control and vascular complications alerting. Non-invasive method of skin AOA/OA measurement seems to be useful for mass screening of hypertensive people for treatment control and early alerting of vascular complications. The further clinical studies for establishment of correlation between the AOA/OA and vascular complications in patients with arterial hypertension are needed and seems to be perspective.

4. CONCLUSIONS

The development of new sensitive non-invasive methods capable to detect signs of a disease at its earliest stages is of great importance. Oxidative stress is one of the criteria of health problems, be they latent or overlooked.

The causes of oxidative stress are a misbalance in the system of generating ROS and antioxidant protection. The monitoring of the latter can be implemented by more or less complex, usually invasive methods.

The existing interrelation between inner body and skin pathologies makes it possible to use data of the content of antioxidants/oxidants in skin as a source of information about a human organism as a whole. Thus, noninvasive potentiometric method of skin AOA/OA assessment can be considered as an integral parameter or as a tool to receive information about health problems, to identify risk groups, to monitorAOA/OAstatus for preventive health care and decease therapy.

Moreover, these findings may contribute to the development of future clinical and basic studies of skin, treatment of skin diseases and aging, to evaluate effectiveness of cosmetic creams. The non-invasive method of skin AOA/OA can be used in screening of hypertensive people for control of compliance with antihypertensive therapy and early alerting of possible vascular complications in such kind of patients.

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