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STUDIES OF ALTERATIONS OF THE CELLULAR MEMBRANE BARRIER FUNCTION AT LARYNGEAL CANCER

Introduction. *Cellular membrane barrier alterations lead to metabolic and functional disorders. However, in the case of laryngeal cancer (LC) they are insufficiently studied.*

The aim of the study *– to learn the nature of the interaction of erythrocyte membranes with introduced spin probes as an indicator of changes in the barrier function of membranes at LC.*

Research Methods. *Samples of the erythrocyte membranes from 40 patients with LC stages II and III and 20 healthy volunteers were probed by EPR with AdTEMPO test. Microviscosity of erythrocyte membranes was determined by the τ_{eff} and the decreasing in RSSI. The content of MWM was identified in the blood plasma and in erythrocyte. The partition coefficient between blood plasma proteins and erythrocyte glycocalyx was calculated. SCEM was evaluated by amount of unabsorbed methylene blue.*

Results and Discussion. *It was established that LC patient's endogenous intoxication is characterized by excessive accumulation of the total pool of MWM both in blood plasma and glycocalyx of erythrocyte. SCEM was significantly decreased in samples of both LC stages in comparison to control. The most apparent decline in τ_{eff} was observed prior to washing of erythrocytes for 5 min after probe insertion. The deceleration after 60 min was observed only in LC stage II. The value of τ_{eff} was at control values levels after washing of erythrocytes of LC stage II 5 min after probe insertion and was significantly reduced in stage III LC in comparison to control. RSSI in samples both stage of patients prior to and after washing of erythrocytes was on average 1.5-fold higher than that of control.*

Conclusions. *It was established that the LC patient's endogenous intoxication is characterized by excessive accumulation of the total pool of MWM both in blood plasma and glycocalyx of erythrocytes, activation of catabolic processes in plasma, redistribution of MWM between the pool of erythrocyte proteins, which corresponds to the second stage of endotoxicosis. The reduction of the SCEM is shown, which is a manifestation of pathological changes in the surface functional activity of erythrocyte membranes. The effectiveness of AdTEMPO for the evaluation of microviscosity of erythrocyte membranes in patients with LC was confirmed.*

KEY WORDS: **laryngeal cancer; erythrocytes; middle weight molecules; sorption capacity of erythrocyte membranes; EPR; nitroxyl radicals.**

INTRODUCTION. Among the various functions of cell membranes one of the most important is the barrier one. It is not limited by a simple separation of the cytoplasm from the extracellular environment, however, it provides also the selective passive and active metabolism with the environment. In addition to supplying the cell by the necessary substances and removing catabolites, the selective permeability of the membranes prevents the entry of unwanted substances into the cell. The fulfillment of these needs, and many other tasks, is ensured by the evolutionarily developed mechanisms with participation of lipids, proteins, carbohydrates. Their functions inside membranes are in close interaction both with each other and with intracellular and extracellular substances. Obviously, that the introduction of foreign substances into the membrane cannot but violate this consistency. Among the variety of such substances the protein and peptide components of metabolic intoxication are leading both by the frequency of manifestation and by the variety of complications caused.

Metabolic intoxication is an integral part of the various diseases that are associated with the impairments of the normal metabolism [1]. Furthermore, the noticing fact is that among of secondary toxins, the key role is played by middle weight molecules (MWM), that are a wide group of substances of predominantly protein-peptide nature [2]. In majority they are produced by non-functional

proteolysis, which is an integral part of the inflammatory processes of any etiology [3]. Unlike the original molecules, the structure of such fragments is unbalanced, which makes them capable for interacting with cell membranes and incorporating into the latter [4]. Such membrane inclusions are able to aggregate both between themselves and with integral membrane proteins. As the result, the conformational mobility of these membrane components undergoing changes with the following functional insufficiency of these proteins [5]. No less sufficient are the regularities of the protein structures formation at influence of a phospholipid bilayer of the membrane (membrane folding). They lead to the exposure of the outer surface of the membrane and activation of proteolysis and the reaction of the immune system. The parts exposed on the intracellular surface of the membrane are formed according to the "positive inside" rule and are enriched by imbalanced positively charged amino acid residues, which can be ideal substrates for non-enzymatic glycosylation [6]. All these processes affect significantly the functioning of the various components of the cell membrane and the cell as a whole [7]. Though, in which measure can all these factors interfere on the barrier function of membranes? The solution of this question seems to be of peculiar importance at cancer research. Owning to the fact that the answer is connected both with the development of endogenous intoxication and with the ability of cancer cells to sorb and accumulate the nanoparticles of diverse nature [8]. On the other hand, the violation of the barrier function of cell membranes can be the cause of chromosomal aberrations inherent in cancer cells [9]. The red blood cells informatively reflect the state of cell membranes at the diseases of various etiology and localization [10, 11]. Therefore, the aim of this work was to study the nature of the interaction of erythrocyte membranes with introduced spin probes as an indicator of membrane barrier function changes at laryngeal cancer (LC).

RESEARCH METHODS. We investigated 40 patients of Kolomiychenko Institute of Otolaryngology (Kyiv, Ukraine). Male patients aged 45 through 65 years with initial stages of LC were selected for participation. Of them, 20 patients were diagnosed with laryngeal keratotic squamous cell carcinoma ($T_2N_0M_0$) stage II and 20 – with ($T_3N_0M_0$) stage III. The control group was composed of 20 apparently healthy volunteers. All the groups were randomized for age and sex composition. All subjects were informed about the aim of the study. Informed consent was obtained from every participant.

Blood samples were obtained from median cubital vein puncture of fasting patients in the morning and mixed with 3.8 % sodium citrate anticoagulant (9:1) in plastic test tubes. Platelet-poor plasma and erythrocyte mass were obtained by conventional methods of selective centrifugation [12]. The content of MWM were identified in the blood plasma and in erythrocyte in an SF-26 spectrophotometer [13]. It was expressed in terms of conventional units (CU), that were equal to the optical density of the solution, which were measured at a wavelength of 242, 254, and 280 nm [14]. The partition coefficient between blood plasma proteins and erythrocyte glycocalyx was calculated by the formula (1):

$$K_1 = \frac{(E_{242}+E_{254}+E_{282})\text{plasma}}{(E_{242}+E_{254}+E_{282})\text{erythrocytes}},$$

Sorption capacity of erythrocyte membranes (SCEM) was assayed after A. A. Togaybayev with modifications by T. V. Kopytova [15]. The amount of unabsorbed methylene blue was evaluated, and the amount of absorbed dye was calculated according to the following formula (2):

$$A(\%) = \left(100 - \frac{E_e}{E_c}\right) \times 100\ \%,$$

where E_e is the experimental sample absorbance and E_c is the control sample absorbance.

The electron paramagnetic resonance (EPR) studies were performed on erythrocytes prior to and post-triple washing with 0.9 % sodium chloride solution. Microviscosity of erythrocyte membranes was studied by spin probe method using lipophilic adamantane-based nitroxyl radical – bis(1-oxyl-2,2,6,6-tetramethylpiperidin-4-yl) ester of 5,7-dimethyladamantane-1,3-dicarboxylic acid (AdTEMPO). This compound was shown to be able to incorporate in a model lipid bilayer, with nitroxyl group localizing between water and lipid phase (Fig. 1) [16, 17].

Final probe concentration in samples was $5 \cdot 10^{-4}$ M. EPR spectra were registered with Varian E-3X-band spectrometer (9 GHz). The strength of center magnetic field was 3210 G, a time constant 1 s, scan time 4 min. We used glass capillary probes of 0.1mL with the inner diameter of 3mm. The simultaneous standard signal of Mn^{2+}/MgO (3 and 4 lines) with known values of *g*-factor was used to equalize experimental conditions.

The following parameters from the obtained spectra were calculated: the effective rotational diffusion correlation times (τ_{eff}), hyperfine interaction

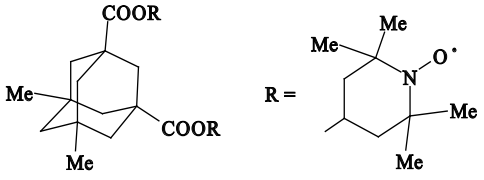

Fig. 1. Structure of the AdTEMPO.

constants (a), and radical spectrum signal intensities (RSSI). Correlation time for rotational diffusion was calculated based on the following formula (3):

$$\tau_{eff}=6{,}65\cdot10^{-10}\Delta H_{+1}\left(\sqrt{\frac{I_{+1}}{I_{-1}}}-1\right),$$

where I_{+1}, I_{-1} are the intensities of the low-field and high-field lines and ΔH_{+1} is the peak-to-peak line width of low-field line.

Statistical processing was carried out using WinPEPI package of programs for biometrics research. For the parameters that corresponded to the normal distribution (according to the results of the Shapiro-Wilk test), the Student's t-test was used, taking into account the recommendations of Gubler [18] and Maltsev [19]. From the data set, the arithmetic mean of the variation series (M), the error of the arithmetic mean (m) was calculated. Mann – Whitney U-test were used to assess differences between the data on LC of different stages and control group. The description was made with the mean and the standard deviation for the parameters that followed a normal distribution, and the median (P_{50}) and 25 (Q_1) and 75 (Q_3) percentiles for the variables that did not follow a normal distribution. Differences were considered significant at $p\leq0.05$.

RESULTS AND DISCUSSION. According to the results obtained, the content MWM in plasma and in erythrocytes of the groups of patients with cancer and healthy persons one was remarkably different (Table 1).

The Table 1 shows the growth trend of MWM in the blood plasma of patients with LC stage II, moreover, at the stage III group this index was statistically sharply increased. ($p<0.01$). The differences in the content of MWM in blood plasma at various states of probably depends on their distribution between plasma and erythrocytes. Due to these facts, we determined MWM content both in blood plasma and in erythrocytes. To evaluate the informative value of these indices, the coefficient of the distribution of MWM between blood plasma and the erythrocyte glycocalyx was calculated. Its difference between the groups of patients with LC stages II and III both relatively to the control group was significant 1.7 and 1.4 times, respectively ($p<0.01$ and $p<0.05$). Such difference may be caused by an increase of catabolic processes in tumor with following release of the toxic products into the bloodstream. At the same time, the increase in MWM content in erythrocytes may be as the result of adsorption on the surface of erythrocytes as by their formation by erythrocytes themselves [20].

Evaluation of the sorption capacity is widely applied to assess state of erythrocyte membranes. SCEM analysis provides information on reduction capacity of erythrocytes, which changes with fluctuation in plasmalemma barrier functions. SCEM in patients with stage II of LC was detected in 40% of the patients and was on average significantly lower in comparison to control (Table 2).

It was not measured in 60 % of the patients of this group. SCEM in patients with LC stage III was detected in 33 % and was on average also low in comparison to control group ($p<0.001$). It was not measured in 67 % of the patients of this group.

Diminished sorption capacity is interpreted by some authors as an indicator of energy deficiency in the erythrocyte. Thus, degradation of erythrocyte membranes and trans-membrane transport leads to disruptions in energy metabolism within the erythrocytes accompanied by decreased ATP content and pyruvate kinase activity [21]. Hence, we established the decrease in SCEM from blood of LC patients in comparison to healthy controls, irrespective of the stage of the disease. Notably, the difference between both experimental group measurements was insignificant. We consider the

Table 1 – **The content of MWM in blood plasma and in erythrocytes of patients with laryngeal cancer and healthy persons**

Indices	Healthy volunteers, n=20	LC patients	
		Stage II, n=20	Stage III, n=20
MWM, CU	0.135±0.003	0.145±0.005	0.160±0.009*
Index of MWM distribution between blood plasma proteins and erythrocyte glycocalyx, CU	0.37±0.05	0.63±0.04*	0,52±0.05*

Note. * – the validity of the difference between the indices of the studied groups.

Table 2 – **Sorption capacity of erythrocyte membranes of laryngeal cancer patients**

Experimental Groups	Mean; P_{50};Q1-Q3
Healthy volunteers n=20	45.89; 50.21 (43.85; 53.68)
Patients with LC stage II n=10	2.35; 0.92 (0.4; 4.3)*
Patients with LC stage III n=11	2.03; 1.63 (0.23; 4.22)*

Note. * – the validity of the difference between the indices of the studied groups.

downward of SCEM in blood of the patients as an indicator of pathological changes in surface functional activity of erythrocyte membranes, in particular of their inability to efficiently transport metabolites via bloodstream [21, 22].

EPR spectroscopy is a valuable technique allowing for detection of changes in cellular metabolic processes. The spin probes, in particular, can be used to study structural changes in cellular membranes at early stages of metabolic disruptions [23]. Nitroxide radicals are the most widely used spin probes. In the first instance we measured hyperfine interaction constants (a) from EPR spectra of the radical in erythrocyte suspension of all the groups in order to characterize spin probe environment prior to the experiments. It was measured to be 16.75 G in all instances, which indicates that AdTEMPO embeds, incorporate into lipid bilayer of erythrocyte membrane with nitroxyl fragment situated on the lipid-water phase boundary. Mobility of the biradical in membrane environment was expressed as effective rotational diffusion correlation time (Table 3).

Since various substances may be absorbed by cellular membranes and block their receptors, increasing their lability and disrupting permeability, all the experiments were performed on erythrocytes prior to and after washing with 0.9 % sodium chloride solution. Probe diffusion was slow in samples of erythrocytes from blood of patients with LC stages II and III of characterized by τ_{eff} at 5 min after the probe's introduction ($p<0.05$). The biradical mobility had a tendency to decrease in 60 min in patients with LC stage II in comparison to control. The difference of this parameter in patients with LC stage III in comparison to control and the other experimental group was not significant.

The value of τ_{eff} in LC stage II patient group after erythrocytes washing was close to control levels with 5 min after probe introduction. We observed a tendency to increased mobility of the biradical in erythrocytes after washing. At stage III, this parameter was significantly higher than that of control ($p<0.02$), indicating slower probe dissipation. No difference was detected between data prior to and post washing. The value of τ_{eff} had a tendency to be higher in patients at stage II after 60 min exposition. At stage III this parameter was not significantly above that of control due to notable differences between individual readings.

Erythrocyte membrane permeability in all groups was also evaluated after the rate of radical spectrum signal drop per hour (Fig. 2).

At stages II and III of the pathology the residual signal spectrum after erythrocyte washing was on average of 1.5 times lower in comparison to control ($p<0.01$ and $p<0.001$, respectively). After washing, this parameter in groups with stage II and III of LC increased statistically significantly in comparison to control in equal, although lesser, measure (1.4 times on average) ($p<0.02$ and $p<0.01$). There was slightly variance between signal spectra before and after erythrocyte washing by 0.9 % sodium chloride.

According the results with the spin probe, diffusion may be interpreted as happening in two stages: membrane surface sorption and permeation into lipid bilayer. We can thus observe various effective rotational diffusion correlation times for binding of AdTEMPO to membrane surface (5 min) and during the probe permeation of erythrocyte membranes (60 min). Primary sorption of AdTEMPO on erythrocyte membranes is associated with increased τ_{eff}, which indicates inhibited torsion of the probe. The effective rotational diffusion correlation time further increases (in 60 min), yet these

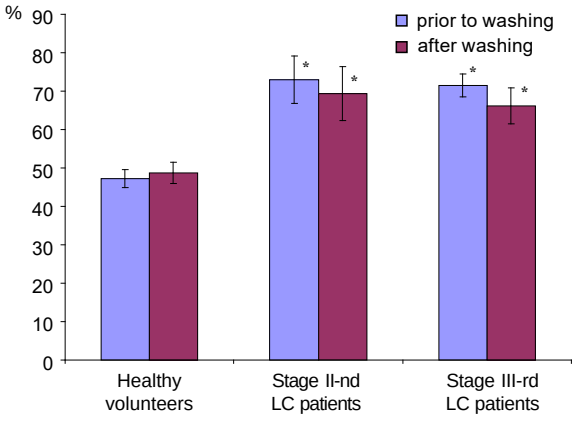

Fig. 2. RSSI in erythrocytes of LC patients prior to and post-triple washing with 0.9 % sodium chloride solution.

Table 3 – **Values of τ_{eff} in erythrocyte membrane environment of patients with LC prior to and post washing with 0.9 % sodium chloride solution**

Experimental Groups	Erythrocytes prior to washing $\tau_{eff} \cdot 10^{-10}$, s		Erythrocytes after washing $\tau_{eff} \cdot 10^{-10}$, s	
	5 min	60 min	5 min	60 min
Healthy volunteers	4.69±0.16	5.09±0.18	4.56±0.13	5.01±0.20
Patients with LC stage II	5.95±0.51*	5.83±0.30	4.18±0.69#	5.59±0.20
Patients with LC stage II	5.26±0.23	5.62±0.22	5.17±0.17*	5.50±0.15

Note. * – significance of difference between healthy group and patients group data; # – significance of difference between data prior to and post washing with 0.9% sodium chloride solution in the same group.

changes are not substantial, which may result from disruptions in lipid bilayer structure and integrated proteins [24, 25]. This assumption was confirmed by data of residual signal intensity. Cellular membrane permeability decreases significantly in laryngeal cancer patients, and this bars intracellular antioxidants for interacting with nitroxyl radical that is on the outer surface of the plasmalemma. The cellular membrane of the healthy volunteers is intact and functions normally.

There is a certain analogy in the development of malignant tumors and bacterial biofilms [26]. An important role in the formation of the latter belongs to horizontal gene transfer. Apparently, in the case of malignant neoplasms, such a transfer is facilitated by a violation of the barrier function of the cell membranes, which are located in the local focus of accumulation of endogenous intoxication products. In this case the damaging effect of MWM will be much more pronounced than in the case of erythrocyte membranes and play a significant role in the malignant process.

CONCLUSIONS. Taking into account the abovementioned, we may finally draw the conclusion, that in LC patients endogenous intoxication is characterized by excessive accumulation of the total pool of MWM both in blood plasma and glycocalyx of erythrocytes, activation of catabolic processes in plasma, redistribution of MWM between the pool of erythrocyte proteins, which corresponds to the second stage of endotoxicosis. The reduction of the SCEM is shown, which is a manifestation of pathological changes in the surface functional activity of erythrocyte membranes, in particular their inability to transport metabolites by blood circulation. The effectiveness of the nitroxyl radical AdTEMPO for the evaluation of microviscosity of erythrocyte membranes in patients with LC has been confirmed. The results of the work indicate the importance of investigating the indicators of the status of erythrocyte membranes in patients with LC. The investigation of our proposed indicators can help to find criteria for dynamic monitoring of the condition of patients with this pathology to improve the assessment of their condition. At the same time, our data suggest the involvement of molecules in impaired membrane function, which can lead to a characteristic of the malignant genetic aberration process.

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ІНСТИТУТ ОТОЛАРИНГОЛОГІЇ ІМ. ПРОФ. О. С. КОЛОМІЙЧЕНКА НАМН УКРАЇНИ[1], КИЇВ
ІНСТИТУТ БІООРГАНІЧНОЇ ХІМІЇ ТА НАФТОХІМІЇ ІМ. В. П. КУХАРЯ НАН УКРАЇНИ[2], КИЇВ

ДОСЛІДЖЕННЯ ЗМІН БАР'ЄРНОЇ ФУНКЦІЇ КЛІТИННИХ МЕМБРАН ПРИ РАКУ ГОРТАНІ

Резюме

Вступ. *Зміни бар'єрної функції клітинних мембран при раку гортані (РГ) призводять до метаболічних і функціональних порушень, які недостатньо вивчено.*

Мета дослідження *– вивчити характер взаємодії мембран еритроцитів зі спіновими зондами як індикатор змін бар'єрної функції мембран при раку гортані.*

Методи дослідження. *Зразки від 40 пацієнтів з РГ II і III стадій та 20 здорових осіб досліджували методом електронного парамагнітного резонансу, використовуючи зонд AdTEMPO. Мікров'язкість мембран еритроцитів визначали за часом кореляції обертальної дифузії (τ_{eff}) і зниженням інтенсивності сигналу спектра радикала. Визначали вміст молекул середньої маси в плазмі крові й еритроцитах і розраховували коефіцієнт розподілу між протеїнами плазми крові та глікокаліксом еритроцитів. Сорбційну ємність еритроцитів оцінювали за кількістю неабсорбованого метиленового синього.*

Результати й обговорення. *Встановлено, що при РГ ендогенна інтоксикація пацієнтів характеризується надлишковим накопиченням загального пулу молекул середньої маси як у плазмі крові, так і в глікокаліксі еритроцитів. Сорбційна ємність еритроцитів при обох стадіях РГ була знижена порівняно з контролем. Найбільш значне зменшення τ_{eff} спостерігали до відмивання еритроцитів через 5 хв після введення зонда. Уповільнення τ_{eff} через 60 хв відзначали тільки при II стадії РГ. Після відмивання еритроцитів при II стадії РГ через 5 хв після введення зонда τ_{eff} перебував на рівні контрольних значень і значно зменшувався при III стадії порівняно з контролем. Інтенсивність сигналу спектра радикала у зразках хворих на РГ обох стадій до і після відмивання еритроцитів у середньому в 1,5 раза була вищою, ніж у контрольній групі.*

Висновки. *Встановлено, що у хворих на РГ ендогенна інтоксикація характеризується надлишковим накопиченням загального пулу молекул середньої маси як у плазмі крові, так і в глікокаліксі еритроцитів, активацією катаболічних процесів у плазмі, перерозподілом молекул середньої маси між пулом протеїнів еритроцитів, що відповідає II стадії ендотоксикозу. Показано зниження сорбційної ємності еритроцитів, що є проявом патологічних змін поверхневої функціональної активності мембран еритроцитів. Підтверджено ефективність зонда AdTEMPO для оцінки мікров'язкості мембран еритроцитів у пацієнтів з РГ.*

КЛЮЧОВІ СЛОВА: **рак гортані; еритроцити; молекули середньої маси; сорбційна ємність мембран еритроцитів; електронний парамагнітний резонанс; нітроксильні радикали.**

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ИНСТИТУТ ОТОЛАРИНГОЛОГИИ ИМ. ПРОФ. О. С. КОЛОМИЙЧЕНКО НАМН УКРАИНЫ[1], КИЕВ
ИНСТИТУТ БИООРГАНИЧЕСКОЙ ХИМИИ И НЕФТЕХИМИИ ИМ. В. П. КУХАРЯ НАН УКРАИНЫ[2], КИЕВ

ИССЛЕДОВАНИЕ ИЗМЕНЕНИЙ БАРЬЕРНОЙ ФУНКЦИИ КЛЕТОЧНЫХ МЕМБРАН ПРИ РАКЕ ГОРТАНИ

Резюме

Вступление. *Изменения барьерной функции клеточных мембран при раке гортани (РГ) приводят к метаболическим и функциональным нарушениям, которые недостаточно изучены.*

Цель исследования *– изучить характер взаимодействия мембран эритроцитов со спиновыми зондами как индикатор изменений барьерной функции мембран при раке гортани.*

Методы исследования. *Образцы от 40 пациентов с РГ II и III стадий и 20 здоровых лиц исследовали методом электронного парамагнитного резонанса, используя зонд AdTEMPO. Микровязкость мембран эритроцитов определяли по времени корелляции вращательной диффузии (τ_{eff}) и снижению интенсивности сигнала спектра радикала. Определяли содержание молекул средней массы в плазме крови и эритроцитах и рассчитывали коэффициент распределения между протеинами плазмы крови и гликокаликсом эритроцитов. Сорбционную емкость эритроцитов оценивали по количеству неабсорбированного метиленового синего.*

Результаты и обсуждение. *Установлено, что при РГ эндогенная интоксикация пациентов характеризуется избыточным накоплением общего пула молекул средней массы как в плазме крови, так и в гликокаликсе эритроцитов. Сорбционная емкость эритроцитов при обеих стадиях РГ была снижена по сравнению с контролем. Наиболее значительное уменьшение τ_{eff} наблюдали до отмывания эритроцитов через 5 мин после введения зонда. Замедление τ_{eff} через 60 мин отмечали только при II стадии РГ. После отмывания эритроцитов при II стадии РГ через 5 мин после введения зонда τ_{eff} находился на уровне контрольных значений и значительно уменьшался при III стадии по сравнению с контролем. Интенсивность сигнала спектра радикала в образцах больных РГ обеих стадий до и после отмывания эритроцитов в среднем в 1,5 раза была выше, чем в контрольной группе.*

Выводы. *Установлено, что у больных РГ эндогенная интоксикация характеризуется избыточным накоплением общего пула молекул средней массы как в плазме крови, так и в гликокаликсе эритроцитов, активацией катаболических процессов в плазме, перераспределением молекул средней массы между пулом протеинов эритроцитов, что соответствует II стадии эндотоксикоза. Показано снижение сорбционной емкости эритроцитов, что является проявлением патологических изменений поверхностной функциональной активности мембран эритроцитов. Подтверждена эффективность зонда AdTEMPO для оценки микровязкости мембран эритроцитов у пациентов с РГ.*

КЛЮЧЕВЫЕ СЛОВА: **рак гортани; эритроциты; молекулы средней массы; сорбционная емкость мембран эритроцитов; электронный парамагнитный резонанс; нитроксильные радикалы.**

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