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Peculiarities of changes in parameters of component body composition of representatives of the youth age period detected by the method of bioimpedance analysis

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Abstract

The results of the study of body composition parameters using bioimpedance analysis of representatives of the youth age period of 14–19 years are presented. The obtained data of bioimpedance analysis for this age category are in satisfactory agreement with the literature data. It is determined that the intracellular liquid of individuals, related to the total volume of liquid in it, increases with age of the individual. It has been suggested that the amount of intracellular liquid relative to the total liquid increases due to an increase in the number of cells (since tissue growth occurs due to active cell division) as well as decrease in the number of extracellular liquid, most often caused by an increase in fatty tissue for this age group. This pattern is observed for female and male contingent of the studied. The distributions of relative intracellular liquid depend on age and change from approximately uniform in adolescent to normal in youthful age. The specific basal metabolism is increasing for males between the ages of 14 and 19, which is explained by the increasing secretion of hormones of the anterior pituitary (somatotropic hormone or growth hormone). The hormone causes a pronounced acceleration of linear growth and, as a result, an increase in the specific basal metabolism in the puberty, especially in males.

Аннотация

Приведены результаты исследования параметров состава тела методом биоимпедансного анализа у представителей подросткового и юношеского возрастных периодов 14–19 лет. Полученные собственные данные биоимпедансного анализа данной возрастной категории удовлетворительно согласуются с литературными. Определено, что внутриклеточная масса биологических объектов, отнесенная к общему объему жидкости в нем, растет с увеличением возраста объекта. Высказано предположение, что количество внутриклеточной жидкости по отношению к общей жидкости в рассматриваемой возрастной группе возрастает за счет увеличения количества самих клеток (поскольку рост тканей происходит за счет активного деления клеток), а также благодаря уменьшению количества внеклеточной жидкости, причиной которого чаще всего является увеличение количества жировой ткани. Эта закономерность наблюдается как для женского, так и мужского контингента исследуемых. Распределения относительной внутриклеточной жидкости существенно зависят от возрастной категории и изменяются от приблизительно равномерного в подростковом возрасте до нормального в юношеском. Удельный основной обмен для лиц мужского пола в возрасте от 14 до 19 лет нарастает, что объясняется нарастающей секрецией гормона передней доли гипофиза (соматотропного гормона или гормон роста). В пубертатном периоде, в особенности у лиц мужского пола, он вызывает выраженное ускорение линейного роста и, как следствие, повышение удельного основного обмена.

Keywords: Component composition, bioimpedancemetry, youth age

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1. Introduction

All currently used methods for assessing the component composition of the body are classified into reference methods, laboratory and field ones [1-2]. The reference methods include multicomponent models, computerized tomography (CT), magnetic resonance imaging procedure (MRI). Laboratory methods consist of dual energy X-ray absorptiometry, densitometry, hydrometry, ultrasonic investigation (ultrasound), three-dimensional scanning. Field methods include anthropometry, bioimpedansometry, determination of body mass index. Anthropometric methods are the simplest and most affordable methods based on changes in the morphometric parameters of the body. Based on these indicators the method of calculating body mass index (BMI) or the Kettle index, is used to assess excess or underweight. However, the BMI indicator is the only a factual sign of the presence of excess or underweight, without giving a qualitative assessment of the body components. For this reason, the biophysical method of studying the component composition of the body, founded in 1880, gained great popularity. W. Thomson, who studied the resistance of tissues of individuals , proposed the method. He suggested that in addition to the general resistance inherent in the body, it is possible to determine the resistance of its individual components. The electric resistance of biological tissues which called bioelectric impedance, and the method itself is called the bioimpedance analysis method. The total impedance value includes two components: the active resistance (or tissue inherent resistance) and reactance, characterized by a shift in the phase of the current relative to the voltage due to the capacitive properties of cell membranes. The total water content in the body is calculated by the value of active resistance. The high conductivity of water is due to the presence of electrolytes in it. The values of the main metabolism and active cell mass, namely the mass of muscles and internal organs are calculated by the magnitude of the reactive component of the impedance. The advantages of the method are low cost and availability, the absence of radiation exposure, the ability to conduct studies in dynamics, as well as the non-invasiveness of the method and the ability to perform in the field.

Currently, the bioimpedance analysis method is used in a wide range from examining the parameters of the human body condition, including medical aspects [1–5] to attempts to determine the biological age of an individual and the nutritional status [6–8]. A number of literature reviews [7–11] present an analysis of modern literature regarding the potential of the bioimpedance method in assessing of the human body composition.

The bioimpedance analysis method (BIA) based on measuring the electric conductivity of biological tissues, allows to evaluate the morphological and physiological parameters of the body in a wide range. In the BIA the active and reactive resistance of the body (or its segments) are measured at frequencies from 5 to 500 kHz. Using the numerical values of the obtained resistances in combination with anthropometric data (mass, height, gender and age of the individual), such characteristics of the individual composition as fat, cell and musculoskeletal mass, volume and distribution of extra- and intracellular liquid in the body are calculated [1]. A special equipment called a bioimpedance meter is used to conduct BIA. Currently, BIA is successfully used by physichians of various specialties (nutritionists, endocrinologists, cardiologists, sports medicine and etc.) in their practice [11].

The bioimpedance measurements show that there are significant differences in the composition of the human body, depending on the age and gender [1, 6, 8, 11]. It has been established that for women in the range from youthful to old age, fat mass monotonously increases, however, its decrease is noted at senile age [1]. The proportion of lean mass and its individual components (musculoskeletal, total liquid volume, intracellular and extracellular liquid) simultaneously decrease with an increase in fatty tissue in the body. The lean mass is up to 80% of body weight in adolescent and youthful categories of women (16–20 years old), and its decline starts from the first period of adulthood (21-35 years old), and it becomes less on 10% in the second adulthood (36-60 years old) than in adolescence. A complete decrease in lean mass from youthful to old age is 15%.

The study of the dynamics of body parameters in children is of particular relevance for dynamic monitoring of the development of the body [8, 12, 13, 16, 18]. The results of studies conducted by anthropologists and pediatricians show the possibility of using BIA in child anthropology, starting at the age of 10. One of the directions of modern research is the study of body composition in youth age periods [5, 14, 18, 19], when the formation of the main parameters of the human body occurs. The puberty occuring during this stage is a process of changes in the body of adolescents, as a result of which they become adults and able to continue the generation. Despite individual differences, the average pubertal period begins at 12-14 years and ends at 18-20 years. Puberty is triggered by signals from the brain through the pituitary-hypothalamus system to the gonadotropic hormones, testes and ovaries. The gonadotropic produce various hormones that stimulate the growth and development of the brain, bones, muscles, skin, and reproductive organs in response to these signals. The growth of the tubular bones of the skeleton accelerates in the first half of puberty and ends completely with the completion of puberty. Prior to puberty, differences in the structure of the body of a girls and boys are reduced exclusively to primary sexual characteristics. The significant differences in the size, shape, composition and function of many structures and systems of the body are related to secondary sexual characteristics during the period of puberty. For a given age period (14-19 years), the physical development takes place in human ontogenesis, which is considered as a dynamic process of growth (increase in length and body mass,

development of organs and body systems, etc.) and biological maturation of a given individual. The development process of morphological and functional properties of the organism (growth rate, mass gain, a certain sequence of increase in various parts of the body and their proportions, as well as the maturation of various organs and systems at a certain stage of development) are mainly programmed by hereditary mechanisms and implemented according to a certain plan optimal living conditions. Physical development reflects the processes of growth and development of the organism at certain stages of postnatal ontogenesis (individual development), when the transformation of the genotypic potential into phenotypic manifestations most clearly occurs. First of all, the human body is biological, organic and natural body, which is characterized by the term "physical condition" or "state of morphofunctional development". The main parameters, properties and qualities of the biological organization of human include the type of constitution (or physique), morphofunctional organization and motor skills.

An analysis of the literature data [5, 14, 18, 19] shows that currently there is sufficient quantitative material for the measured parameters of the bodies of individuals in adolescence and youth age. It has been also stated that these parameters for adolescents and youths are different depending on nationality and place of residence. This indicates the need for a detailed analysis of the accumulated experimental data. This paper analyzes the features of the development and formation of the basic parameters of a person and energy characteristics for people from the age of 14 to 19.

The aim of research is to determine differences in changes of parameters of the human body composition for representatives of the youth age period.

Interest to this age category of individuals is that significant differences in the size, shape, composition and function of many structures and systems of the human body are formed at this age. The hypothalamic-pituitary system of the body that produces somatotropic hormone is responsible for these processes. It is in childhood and adolescence somatotropic hormone (or growth hormone) causes an acceleration of linear (in length) growth. This is due to the growth of the tubular bones of the skeleton. Somatotropin secretion gradually decreases with age. It reaches minimum values at the elderly and second period of mature age. Maximum values are reached during puberty in adolescents. This phenomenon is accompanied by an acceleration of metabolism, and as a result, the active growth of tissues due to cell division.

2. Material and Methods

Volunteers Volunteers of adolescent (school) and youth (junior students) age periods from 14 to 19 years old have been involved in this study. Volunteers were divided into three age groups: 1) adolescent - 14-15 years old, 2) first youthful - 16-17 years, 3) second youthful - 18-19 years old. Written consents to the bioimpedance analysis were obtained from each volunteer (the age group 18–19 years) or from their parents (the age group 14-17 years).

Table 1 | The composition of the volunteers. Состав выборки исследуемых лиц.

Age, years	Gender	Number of participants, man	Total, man
14-15	F	40	76
	M	36	
16-17	F	52	100
	M	48	
18-19	F	61	122
	M	61	

Table 1 presents the age and gender composition of the volunteers.

The studies were conducted by bioimpedance analysis using the MEDASS ABC-01 body water balance analyzer with the software «Sport» [20]. BIA is based on measuring the body's electric resistance (impedance) using a bio-impedance analyzer [1, 2, 15, 16]. In this case, two pairs of disposable bioadhesive electrodes are used in the «arm – trunk – leg» circuit with a probing sinusoidal current of constant frequency and low power (not more than 500–800 μA). Measurements were performed for each volunteer twice. An electric current, depending on the frequency of the probing signal, flows both around the cell (at low frequencies) and through them (at high frequencies). In the used analyzer, the probe signal is supplied at 2 frequencies: f_1 = 5 kHz and f_2 = 50 kHz. It is believed that at the first frequency, the current passes only through the extracellular liquid (analogue of direct current), at the second frequency, the current passes through both the extracellular and intracellular liquids. The main parameters (fatty mass (FM), lean mass (LM) – body mass without fat, musculoskeletal mass (MSM) and active cell mass (ACM), as well as the total water volume (TWV) differentiated into intracellular liquid (IL) and extracellular liquid) are determined based on the measured active and reactive resistances of a person using also anthropometric data. In addition, data on the energy balance of an individual (the basal metabolism (BM) and the specific basal metabolism (SBM)) are given. As a rule [1, 17, 21], regression equations are used to determine the composition of a individual . Regression equations include the anthropometric and electric parameters of the individual . For example, to determine the total water volume in the body m_{TWV} one have to use the regression formula [21, 25, 27] in the form:

$$m_{TWV} = \frac{a_1 L^2}{R_{50}} + a_2 M + a_3 t + A + a_4 \qquad \text{Equation 1}$$

where L – the height of the person, R_{50} – the active resistance at a frequency of 50 kHz, M – mass of the person, t – age, the value of A is determined by the gender of the volunteer.

Table 2 | Electric indicators for humans of different age and gender groups. Электрические показатели у лиц разных половозрастных групп.

Age, years	Gender	j, degree		R_i, Om		X_c, Om		R_0, Om
		own	literary	own	literary	own	literary	
14-15	F	6 ± 1	7 ± 1	673 ± 75	680 ± 80	74 ± 11	70 ± 11	756 ± 79
	M	7 ± 1	7 ± 1	556 ± 54	540 ± 70	67 ± 7	64 ± 7	634 ± 55
16-17	F	6 ± 1	7 ± 1	653 ± 64	655 ± 75	70 ± 8	68 ± 11	736 ± 71
	M	7 ± 1	7 ± 1	530 ± 70	530 ± 70	65 ± 9	63 ± 7	615 ± 78
18-19	F	6 ± 1	7 ± 1	670 ± 71	640 ± 80	75 ± 9	67 ± 11	760 ± 79
	M	7 ± 1	7 ± 1	560 ± 52	520 ± 80	69 ± 7	62 ± 8	646 ± 58

The constants a_i in the regression equation (1) are determined empirically. To select the constants a_i, the component of the individual is measured in other ways (for example, the dilution method [1] is used to determine the numerical value of the total body liquid mass) and the same component is compared in the form of equation (1). In this case, the desired constants ai are determined by minimizing the error.

Equation (1) can be represented through the electric characteristics and anthropometric data of the human body. In this case, it becomes equivalent to the equation:

$$m_{TWV} = a_1 \frac{V}{\rho_e} + a_2 \rho_T V + a_3 t + A \qquad \text{Equation 1*}$$

where V - the volume of the body, ρ_e - the resistivity of the body, ρ_T – density.

It is believed that one of the main indicators characterizing the state of a individual is the phase angle φ [6, 7, 13, 22]. The phase angle characterizes the phase shift of the alternating current relative to voltage, its values characterize the degree of fitness and endurance of the body, the functional state of the cells and the intensity of metabolism. The phase angle φ is determined from the ratio:

$$\varphi = arctg \frac{X_C}{R} \qquad \text{Equation 2}$$

where X_c - reactive, R - active resistance, which are defined for the three-parameter model in the form [21]:

$$R = \frac{R_0 + \omega^2 C^2 R_0 R_i (R_0 + R_i)}{1 + (\omega C (R_0 + R_i))^2} \qquad \text{Equation 3}$$

$$X_C = \frac{\omega C R_0^2}{1 + \omega^2 C^2 (R_0 + R_i)^2} \qquad \text{Equation 4}$$

where C – total capacity of cell membranes, R_i and R_0 – active components of the resistance of intra-and extracellular liquids.

The table processor Microsoft Office Excel 2007 and the statistical package “Statistica for Windows” 6.0 were used for statistical processing of the results. A control on normal (Gaussian) distribution was carried out using the Shapiro-Wilk W-test. It was believed that with the analyzed distribution does not differ from normal. If the distribution corresponded to the normal one, then the arithmetic mean and standard deviation were determined for the measured parameter: .(X) ± σ.

3. Results and Discussion

The active resistance at the frequency of 5 kHz (R_0), the active and reactive components of the impedance at a frequency of 50 kHz (R_i , X_c) and the phase angle (φ) were determined as a result of bioimpedance studies. The numerical values of the presented parameters are given in Table 2 in comparison with the literature data [21].

As can be seen from table 2, the phase angles for the gender and age categories are generally consistent with the data [22]. Active and reactive resistances monotonously fall with an increase in height and their insignificant growth is observed for the age group of 18-19 years in the conducted study.

Regression-coupling equations allow to determine the main parameters of the human body and are compiled based on electric characteristics with known anthropometric parameters. The data analyzed for the considered age and

Table 3 | Parameters of body composition in individuals of different age and gender groups. Параметры состава тела у лиц разных половозрастных групп.

Age, years	Gender	m_{ACM}/m_{LM}, %		BM, kcal/day		SBM, kcal/(day*m²)		m_{II}/m_{TWV}, %
		own	literary [17]	own	literary [24]	own	literary [17]	
14-15	F	55 ± 4	56 ± 3	1305 ± 100	1250 ± 100	820 ± 53	845 ± 30	0,56 ± 0,01
	M	58 ± 3	55 ± 3	1573 ± 152	1420 ± 110	867 ± 39	910 ± 30	0,59 ± 0,01
16-17	F	55 ± 2	57 ± 4	1460 ± 184	1300 ± 100	818 ± 38	835 ± 35	0,57 ± 0,01
	M	58 ± 2	56 ± 3	1677 ± 116	1560 ± 110	868 ± 41	920 ± 40	0,6 ± 0,1
18-19	F	55 ± 2	57 ± 4	1351 ± 86	1380 ± 100	814 ± 41	830 ± 40	0,57 ± 0,01
	M	59 ± 2	58 ± 3	1675 ± 104	1700 ± 120	880 ± 40	930 ± 40	0,60 ± 0,01

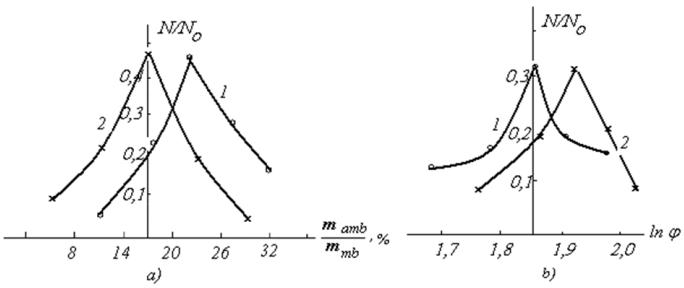

Figure 1 | Distributions by fat mass (a) and natural logarithm of phase angle (b) for adolescents 14-15 years old: 1 – teenage girls, 2 – teenage boys. Распределения по жировой массе (а) и натуральному логарифму фазового угла (б) для подростков 14-15 лет: 1 – девочки-подростки, 2 – мальчики-подростки лаборатории Тюменской области.

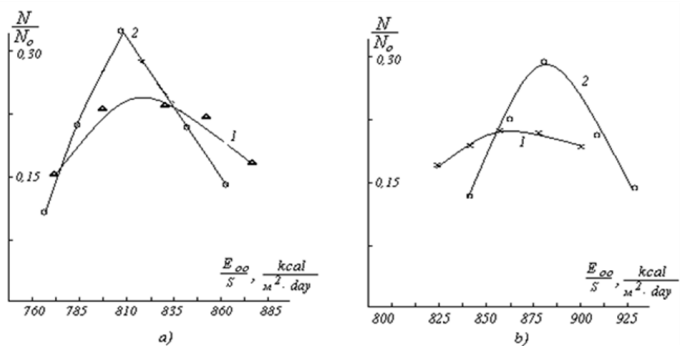

Figure 3 | Distributions by specific basal metabolism: a) – females: 1 – 16-17 years old; 2 – 18-19 years old; b) – males: 1 – 14-15 years; 2 – 18-19 years old. Распределения по удельному основному обмену: а) – лица женского пола: 1 – 16-17 лет; 2 – 18-19 лет; б) – лица мужского пола: 1 – 14-15 лет; 2 – 18-19 лет.

gender categories in the present work are presented in Table 3.

The figure 1 shows the distribution of body fat mass, referred to the mass of the body and from the natural logarithm of the phase angle. In the given data, N0 indicates the total number of investigated individuals (teenage boys or girls), and N the number of investigated, related to the interval of relative fat mass indicated in the figure 1.

As follows from the processing of experimental data, the measurement results can be described by a normal distribution, which can be considered as the evidence of the reliability of the sample. The shifts of the maxima for the presented distributions also correspond to known literature data. It should be noted that distributions are similar for other age and gender categories. There is a high correlation between the relative cell mass (table 3) and the phase angle. The relationship between them is:

$$\frac{m_{ACM}}{m_{LM}} \approx 0.3 \ln \varphi \qquad \text{Equation 5}$$

The figure 2 shows the mass distribution of intracellular liquid, referred to the total volume of the liquid phase in the human body. Such data are not presented in the available

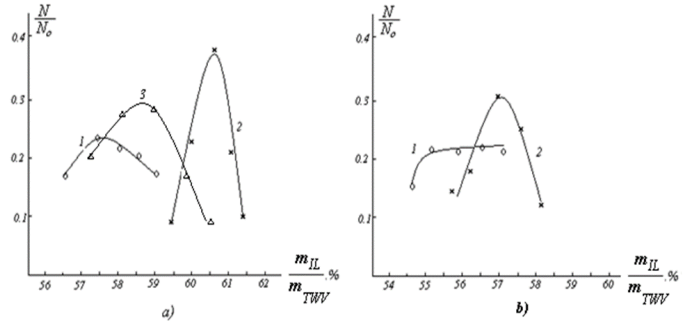

Figure 2 | Distribution of intracellular liquid (IL), related to the total water volume (TWV) in the body: a) – females: 1 – 14-15 years; 2 – 18-19 years old; 3 – 16-17 years old; b) – males: 1 – 14-15 years; 2 – 18-19 years old. Распределения по клеточной жидкости (КЖ), отнесенной к общему водному объему (ОВО) в организме: а) – лица женского пола: 1 – 14-15 лет; 2 – 18-19 лет; 3 – 16-17 лет; б) – лица мужского пола: 1 – 14-15 лет; 2 – 18-19 лет.

literature. In general, it is considered [23-28] that the mass percentage of intracellular liquid is 60% of the total mass of the liquid and depends on the age and gender of the individual.

The data presented on the figure 2 confirm the integral fact, however it follows that during the period from 14 to 19 years, the average relative intracellular liquid also increases from 57.5% to 60.5%. At the same time, the type of distribution for different age and gender categories also changes significantly. If uniform distributions over relative ACM are characteristic for 14–15 years of age, then it assumes the form of a normal distribution for 18–19 years of age. Perhaps, the reason is the pubertal period and the corresponding processes of active growth. There are currently no published data about the interconnection of intracellular liquid with age-related changes, but it can be assumed that the amount of intracellular liquid relative to the total liquid. The total liquid may increase during this period due to two mechanisms: 1) an increase in the number of cells, since tissue growth occurs due to active cell division; 2) a decrease of the amount of extracellular liquid, the cause of which is most often an increase in the amount of fatty tissue, because fatty tissue is little hydrated and there is a direct relation (the higher the number of fat cells themselves, the lower the total body liquid). This pattern is observed for both female and male contingent of the studied.

The figure 3 shows the dependence of the relative number of the studied volunteers from the energy characteristics of a human (SBM). As follows from the literature data (table 3), this characteristic should slowly decrease with increasing age. The obtained experimental data for the female category confirm this fact; however, a contradiction is obtained for the males. The SBM value also increases with age for the given age group.

To explain this phenomenon, it is necessary to investigate the ratio:

$$SBM = \frac{BM}{S} \qquad \text{Equation 6}$$

where S – the body surface area of the individual .

The ACM is associated with the LM ratio (5). Since the phase angle varies insignificantly for the studied age categories, it is obvious that the change in ACM will be determined by the change in the lean mass of the individual , which also increases with age. The BM is associated with an active cell mass ratio:. It follows that, since ACM is increasing, the energy released by a person per unit of time will also increase.

As follows from relation (6), the SBM is determined by two competing values: the growing values of BM and the growing surface square of the body of the individual , defined by the Du Bois formula:

$$S\ (m^2) = 0.007184 * m^{0.425} * L^{0.725}, \text{ or } S\ (m^2) = \sqrt{\frac{m * L}{3600}} \quad \text{Equation 7}$$

where m – is body mass (kg), L – is height (cm).

Calculations of absolute values of S by both formulas coincide with a sufficiently high degree of accuracy. The values of BM and the body surface square of individuals (S) are presented in Table 4.

As follows from table 4, the average mass for the age group of 18-19 years is less than at the age of 16-17 years, while the average growth values exceed. This leads to the fact that the average values of body surface square of categories 16-17-young men exceed the same parameter for 18-19 year olds. This determines the contradictions between the SBM data presented in the literature and the obtained values of the specific basic exchange in our own studies.

4. Concluding Remarks

The intracellular liquid of individuals referred to the total volume of liquid in it depends on the age of the individual . An increase in relative intracellular liquid is observed in the period from 14 to 19 years. It is assumed that the amount of intracellular liquid relative to the total liquid during this period may increase due to an increase in the number of cells themselves, since tissue growth occurs due to active cell division; and also due to a decrease in the amount of extracellular liquid, the cause of which is most often an increase in the amount of fatty tissue, since fatty tissue is a little hydrated. This pattern is observed for both female and male contingent of the studied.

The specific basal metabolism for males increases between the ages of 14 and 19, which can be explained by the increasing secretion of the anterior pituitary hormone (growth hormone) in this category of studied. It causes a marked acceleration of growth in the puberty, especially in males, what confirms the numerous literature data, and, therefore, the reliability of the bioimpedance analysis method in assessing the component composition of the body.

The results of the study can be used to simulate the ways of correction of the components of the body composition and the biophysical parameters of the body based on the identified relationships between them. The possibility of using indicators such as phase angle, active cell mass, specific basal metabolism as indicators of the body's functional state of cells in adolescent and youthful age groups becomes apparent.

The revealed interconnections between the indicators can be used in educational institutions with the aim of monitoring the functional state of the organism of persons of adolescent and youthful age by creating and updating the database.

Заключение

Отношение объёма внутриклеточной жидкости биологических объектов к общему объему жидкости в них зависит от возраста. В период от 14 до 19 лет наблюдается рост относительной внутриклеточной жидкости. Предполагается, что количество внутриклеточной жидкости по отношению к общей жидкости именно в этот период может увеличиваться за счет увеличения количества самих клеток, так как рост тканей происходит за счет активного деления клеток; а также благодаря уменьшению количества внеклеточной жидкости, причиной которого чаще всего является увеличение количества жировой ткани, поскольку жировая ткань мало гидратирована. Данная закономерность наблюдается как для женского, так и мужского контингента исследуемых.

Удельный основной обмен для лиц мужского пола в возрасте от 14 до 19 лет нарастает, что можно объяснить нарастающим выделением именно у данной категории исследуемых гормона передней доли гипофиза (соматотропный гормон или гормон роста), так как именно в пубертатном периоде, а, особенно, у лиц мужского пола, он вызывает выраженное ускорение линейного (в длину) роста и, как следствие, повышение удельного основного обмена, что подтверждает многочисленные литературные данные, а, значит, и

Table 4 | Parameters for calculating the energy characteristics and their values. Параметры для расчетов энергетических характеристик и их значения

Age, years	Mass, m, kg	Height, L, cm	S (m^2)	BM, kcal/day	SBM,
14-15	63 ± 9	178 ± 7	1,8 ± 0,1	1573 ± 152	867 ± 39
16-17	72 ± 9	181 ± 4	1,9 ± 0,1	1532 ± 198	868 ± 41
18-19	70 ± 8	182 ± 7	1,9 ± 0,1	1675 ± 104	880 ± 40

достоверность метода биоимпедансного анализа в оценке компонентного состава тела.

Результаты исследования могут быть использованы для моделирования путей коррекции показателей компонентного состава тела и биофизических показателей тела на основе выявленных взаимосвязей между ними. Становится очевидной возможность использования таких показателей, как фазовый угол, активная клеточная масса, удельный основной обмен как показателей функционального состояния клеток у подростковой и юношеской возрастных групп.

Выявленные взаимосвязи между показателями могут быть использованы в учебных заведениях с целью мониторинга функционального состояния организма лиц подросткового и юношеского возраста путем создания, периодического пополнения и обновления базы данных.

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Regarding the mechanisms of the changes of acetyl cholinesterase (ACE) enzyme activity from erythrocyte membrane under the action of pesticides

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ABSTRACT

The kinetics and mechanisms of the action of some pesticides, such as pentachloronitrobenzene, trichloracetic acid, rogor, sodium pentachlorphenol, chlorophos, heptachlor and photodynamic herbicides on acetylcholinesterase activity and mechanical stability of red blood cells under ultrasound action was studied. The degree of change in the resistance of erythrocytes treated with the pesticides to ultrasound action was determined. The data on the altering of structure-function characteristics correlate well with the results of toxic action of these compounds on different biological objects. These results may be used for selective search of the preparations with lower toxicity and higher economical efficiency.

Аннотация

Изучена кинетика и механизмы влияния некоторых пестицидов: пентахлорнитробензола-ПХНБ, трихлоруксусной кислоты (ТХУ), рогора, пентахлорфенолата натрия (ПХФ-Na), хлорофоса, гептахлора и фотодинамических гербицидов на мембранную активность ацетилхолинэстеразы и механическую стабильность эритроцитов под воздействием ультразвука. Определена степень изменения резистентности обработанных пестицидами эритроцитов к ультразвуку (УЗ). Данные о нарушении структурно-функциональных свойств эритроцитов коррелируют с результатами токсического воздействия этих препаратов на различные биологические объекты. Полученные данные могут быть использованы для селективного поиска препаратов с наименьшей токсичностью и экономической эффективностью

Keywords: Pesticides, ultrasound, acetylcholine, RBC hemolysis

1. Introduction

Pesticides are chemical compounds used for the struggle against pathogenic organisms. World assortment of these preparations is more than 10000 names based on more than 600 chemical compounds of different classes. However, despite of advantages of the chemical method of plant protection there are some disadvantages too. First of all, preparations accumulate in the environment; stable populations of harmful organisms are formed that turn into new resistant species, useful inhabitants of the biosphere suffer greatly, as well as the health of human population [1]. Finally, it leads to the violation of natural biosynthesis in the biosphere. Many authors confirm that high level of morbidity of different etiologies may be caused by the influence of technogenic pollution of the environment on the human health [4]. Humans are under the influence of a huge number of various chemical substances during their life. They enter the body in different ways. Due to the connection with the development of technogenic factors the population of large cities is strongly affected by heavy

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metals like lead, chromium, copper and cadmium and also by aromatic hydrocarbons and pesticides. Compounds of these substances have an adverse effect on the functional state of the digestive system, pancreatic cells, and also cause irritation of the mucous membrane of the small intestine It is confirmed that the increase of chromosomal aberration rate in the lymphocytes of peripheral blood of the persons with chronic lead intoxication is the result of industrial contact with pesticides [1,2]. Presence of induced chromosomal aberrations may be the evidence of the mutagenic action of industrial factors on human population. Thus, the increased rate of mutations in cells of persons and animals contacted with heavy metals has been proved . This process may be the reason of aborts, congenital malformations, hereditary diseases and other pathologies [2]. So, it is necessary to predict the mutagenic danger of substances with which persons may get in contact. Large work is being done in many countries to overcome these disadvantages. Toxic pesticides are replaced by substances with decreased toxicity that are not capable to accumulate in the environment and living organisms. Replacement of DDT (Dichlorodiphenyltrichloroethane) by methyl chloride, dichlor and other insecticides is a very actual point. Such chemical substances promote the changes of blood content and it results in abnormal protein levels in plasma [4,6]. It needs the constant medical check-up. The research of scientists of National Cancer Institute /USA/ on donors showed that pesticides action on organism twice increases the risk of multiple myeloma-malignant cancers of the bone marrow development in the age interval from 30 till 94 . Agricultural employers have been shown to have increased risk of hematological diseases development with a lethal outcome compared to the total human population [6].

The development of effective methods of physical and chemical characteristics of RBC in different pathologies and under the influence of negative factors of environment is one of the important biomedical problems. Fundamental similarity of structural and functional organization of all cell membranes and established total mechanisms of their responses to some pathological conditions make possible to use RBC as a model. That's why it is important to develop the method to study the changes in RBC membranes under various stresses that model certain pathological processes. Application of ultrasound that is used widely for direct and selective effect on cells may give information about mechanical resistance of RBC, since ultrasound can cause a hemolytic effect. Since it is known that pesticides have a damaging effect on cell membranes, one can study the regularities of effects of chlorine-containing pesticides in the model system of RBC suspension. Study of ultrasonic hemolytic resistance of RBC is relevant because pesticides are widely used. The changes of mechanic resistance of RBC treated with pesticides may be used a characteristic of their damaging action on a quantitative level [2].

Getting into the body, drugs may function as powerful blockers of ACE. Apparently, drugs dissolving in the hydrophobic region of erythrocyte membranes, may later form a complex with cytochrome, and leading to the change in membrane stability both directly and indirectly. So, it became necessary to analyze the mechanism of action of ultrasound (US) on RBC membranes, as it has been reflected in our published works [2,3].

There are several mechanisms of the effect of ultrasound on cells and cell suspension listed below:

- the heating (temperature factor);
- chemical damage caused by the action of free radicals;
- mechanical damage caused by shock waves and acoustic flows (mechanic factor).

The purpose of this work was to study kinetics of membrane-bound ACE in presence of chlorine-containing pesticides, as well as to obtain quantitative criteria for comparative evaluation of the action of pesticides.

The dependence of hemolytic activity of some pesticides and herbicides on the level of initial activity of cellular enzymes, on the ability of the drug to reduce membrane fluidity has been studied in a number of works [2,3,5]. However, the kinetic characteristics of the action of chlorinated pesticides on RBC hemolysis and the resistance of RBC to mechanical factors, as well as investigation of certain patterns of physiological activity of these compounds have not been studied yet.

Previously, it was shown that the damaging effect of insecticides in biological systems is directly related to their effect on the lipid phase of biological membranes [3]. However, quantitative criteria for their effect on RBC have not been investigated yet. Research on quantitative regularities of the damaging action of physiologically active compounds (PAC) would be actual.

The aim of this work is to study the kinetics of the action of chlorine-containing pesticides on the activity of membrane-bound ACE-ase, as well as the obtained quantitative criteria for comparative analysis.

2. Material and Methods

As the material we used the suspension of red blood cells isolated from donor blood. The specific activity of ACE was determined by the potentiometric method with automatic registration process. Kinetics of RBC hemolysis in the isotonic medium under the action of pesticides was studied by the photocalorimetric method in suspension (107-108 cells per 1 ml). We used sodium salt of trichloroacetic acid (herbicide), sodium pentachlorophenol (herbicide), trichlorfon (insecticide), pentachloronitrobenzene (fungicide) and rogor (pesticide).

RBC suspension extracted by precipitation from 3 ml of donor fresh blood treated by heparin or citrate (0,5 ml of citrate + 2,5 ml of whole blood) and twice washed from plasma by 0,9% NaCl isotonic solution was used. Centrifugation was performed at 6000 rpm for 10 min (3 times). The washed cells were suspended in 8 ml of saline solution. The suspension of RBC diluted with saline solution

in a ratio of 0,5 ml over 23,5 ml of saline solution (50 times) has been prepared for the research of US hemolysis. Cell concentration of suspension is 30·106 cell/ml. We also carried out a spectrophotometric assessment of mechanical and physical factors (including US fields) kinetics of action on biological membranes. This method allows to investigate the hidden damages of RBC membranes. Resistance of RBC was studied on the basis of method of photometric automatic registration of process of hemolysis of RBC under the influence of continuous ultrasound at the frequency 0,88 MHz in frames of intensity 0,1-1,0 v/sm2 at the constant temperature.

Activity of membrane-associated ACE was determined by the potentiometric method. The substrate was acetylcholine chloride (AC) with initial concentration of 2.5 mM in a measuring cell. To study the kinetics of enzymatic hydrolysis, an incubation mixture was used consisting of a 0.9% NaCl solution containing 2.5 mM Tris-HCl, a suspension of RBC (5-8·10^4 cell / ml), ethanol (no more than 2 % by volume). The speed of reaction in the process of enzymatic hydrolysis of AC has been calculated from experimental kinetic curves at the change of pH of the incubated mixture in control and after the treatment by AC. Incubation time of RBC with preparations was equal to 2 min. Kinetic analysis of the results of the determination of ACE activity of RBC before and after treatment of preparations assessed by estimation of enzyme activity through the change of relative activity (A) (by the tangent of the slope of the kinetic curve) at the action of preparation.

Determination of ACE activity in the suspension of RBC was carried out also by the measurement of the kinetics of the damage of RBC in the process of ultrasound hemolysis (f=0.88 MHz and I=0.3 V/ cm^2). We used the method of US hemolysis that was developed before [2,9]. The speeds of cell mechanical disruption in the sonification was determined by kinetic curves of ultrasonic disintegration before and after treatment by preparations (V_0 and V_1). These indices are the characteristics of mechanic resistance of RBC membranes.

3. Results

In Table 1 one can see structural formulas of substances tested in the current study.

Detected inactivation of the external ACE enzyme indicates that the inhibitory effect of chlorine-containing pesticides is caused by the damage of the membranes of erythrocytes, with which ACE is associated.

As a criterion for assessing the effect of the studied pesticides on the activity of erythrocytes, the concentration of the drug causing enzyme inactivation by 50% (CA_{50}) was used. It was found that in the studied concentrations, these pesticides have certain antibacterial activity (Table 1). Table 1 shows that the studied compounds are not specific acetylcholinesterase inhibitors, as they reduce enzyme activity at significantly higher concentrations (CA_{50} – 120 and 10^{-1} – 10^{-3} mM) than known anti-toxic agents such as phosphine, amiton, etc. (CA_{50} ~ 106-108 mM) [6].

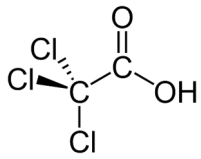
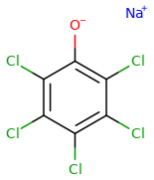
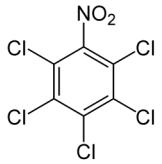
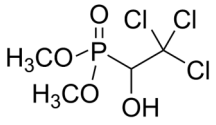
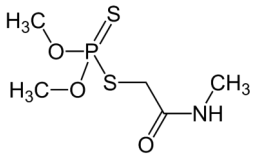
The detected inactivation of the external enzyme (i.e. acetylcholinesterase) suggests that the inhibitory effect of chlorine-containing pesticides is due to the damage of RBC membranes, which are associated with the enzymatic action of ACE [3].

These changes are caused either by the adsorption of the studied pesticides on RBC membranes, or by the inclusion of those substances into the lipoprotein structure of the RBC membrane [7].

One of the extreme manifestations of the modifying effect of chemical compounds on erythrocyte membranes, leading to disruption of cell integrity, is hemolysis. It was found that PCP-Na, chlorophos and rogor possess their own hemolytic activity in isotonic medium, and a treatment of red blood cells by THA-acid and PCNB does not lead to their hemolysis.

From fig 1 and table 2 it can be seen that the hemolytic effect naturally depends on the concentration of the pesticide: with an increase in concentration of the drug in the incubation medium, the hemolysis time t and half-life t50 decreases, and the rate of hemolysis (V) increases accordingly. The method of studying the quantitative

Table 1 | The effect of pesticides on the hemolytic activity of erythrocytes. ффективность пестицидов на гемолитическую активность эритроцитов.

Chemical	Structural formula	CA_{50} (mM)
TCl acid (trichloroacetic acid)		120
PCP-Na (sodium pentachlorophenolate)		$5·10^{-1}$
PCNB (pentachloronitrobenzene)		$2·10^{-2}$
Chlorophos (trichlorphon)		$5·10^{-3}$
Rogor		---

characteristics of hemolysis of red blood cells in the field of ultrasound allows to determine the lowest concentration of the drug that has hemolytic activity in each case.

It can be seen in figure 1 that the kinetic curves are S-shaped with a certain period of induction, and accordingly the rate of hemolysis depends on the concentration of the drug. With the increase in concentration of the pesticide in the incubation medium, the rate of hemolysis increases, and the induction period decreases. The obtained data indicate a strong modifying effect of PCP-Na and chlorophos on the membrane structure of red blood cells, leading to hemolysis of red blood cells.

The resistance of pesticide-treated erythrocytes to mechanical hemolysis under ultrasound was also studied using automatic registration of erythrocyte destruction kinetics in a spectrophotometer cell [2]. In control experiments, it was found that ethanol, used as a solvent for pesticides at a concentration of <0.1% by volume, does not have a noticeable hemolytic effect on erythrocyte cells.

For some pesticides and bactericides that cause hemolysis of erythrocytes in isotonic medium, it has also been shown that along with this, they stabilize erythrocyte membranes to hemolysis in hypotonic medium in certain concentrations (9 μm - 150 μm) [2,5]. It can be assumed that the modifying effect of pesticides on erythrocyte membranes also leads to change in their mechanical resistance to ultrasound. This is consistent with the data on the effect of surfactants on the mechanical stability of erythrocytes [3]. Table 2 presents quantitative indicators characterizing changes in the resistance of erythrocytes to ultrasound exposure under the influence of different concentrations of pesticides, indicating a change in the mechanical stability of cells in the presence of these chemical compounds.

It is also known that pesticides can affect the structure and biological activity of red blood cells. Erythrocytes are convenient model for studies on the damaging effect of various factors, including pesticides, on cell membranes, as well as changes in other membranes [5]. ACE is an enzyme of the outer surface of erythrocyte membranes, brain cells, nerve tissue, etc. and it plays significant role in signal transmission through synapses since it catalyzes the hydrolysis of acetylcholine. ACE activity in blood and its components can serve as an additional diagnostic criterion for the analysis of some pathological conditions caused by toxic compounds. A number of studies have shown the dependence of hemolytic activity of some pesticides on the level of initial activity of cellular enzymes and the ability of the drug to reduce membrane fluidity [6, 7]. However, the kinetic characteristics of the action of a number of pesticides on erythrocyte hemolysis and their resistance to mechanical factors that characterize certain patterns of physiological activity of these compounds, have been previously studied, but insufficiently [8,9]. We have studied the kinetics and mechanisms of the influence of pesticides on ACE activity of red blood cells, namely, the change of erythrocyte resistance to ultrasound exposure in the presence of different concentrations of pesticides.

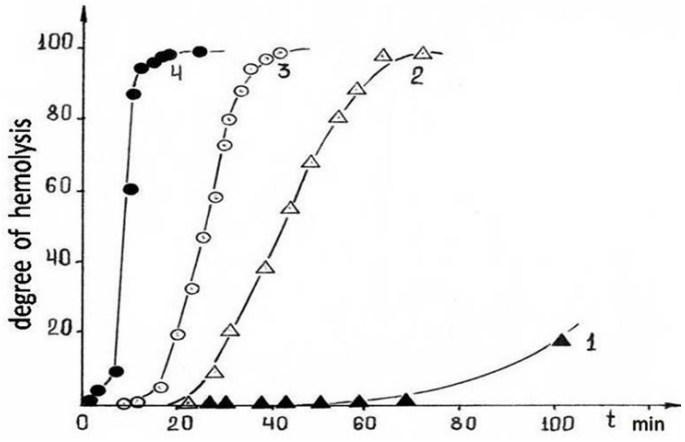


Figure 1 | The Hemolysis of red blood cells under the action of chlorine-containing pesticides, where curve 1is for TCl acid , curve 2 is for chlorophos, curve 3 is for PCP-Na, curve 4 is for rogor. On the abscissa axis there is hemolysis time (resistance) in minutes, on the ordinate axis there is light transmission in %.

Table 2 | The effect of pesticides of different concentration on the parameters of the ultrasound hemolysis and acetylcholinesterase activity in the estimated suspension of erythrocytes (the volume mode continuous, I = 0.4W / cm2, V=0.88 Mgs). Изучение влияния пестицидов различной концентрации на параметры УЗ гемолиза и активность АХЭ-азы в исследуемых суспензиях эритроцитов (режим воздействия УЗ непрерывный, I = 0,4 Вт / см2, V=0,88 МГц).

Samples	Contentration [M]	t hem, c	V hem c^{-1}	$C_{A\,50}$ (mM)
Testing	0	500 ± 10	1,09 ± 0,03	
PCNB	10^{-4} 10^{-3}	700 ± 22 1200 ± 150	0,60 ± 0,02 0,3 ± 0,1	$2{\cdot}10^{-2}$
TClA	(3-6)·10-3 (2-3)·10-2	420 ± 17 360 ± 19	1,12 ± 0,04 1,3 ± 0,1	120
PCP Na	10^{-6} -10^{-5} 10^{-4}-10^{-3} 10^{-2}	450 ± 27 415 ± 46 200 ± 3	1,2 ± 0,1 1,3± 0,1 4,0 ± 0,1	$5{\cdot}10^{-1}$
Heptachlor	10^{-5} 10^{-4}	565 ± 47 830 ± 43	0,97 ± 0,04 0,4± 0,1	
Chlorophos	10^{-3} $5{\cdot}10^{-3}$ 10^{-2}	1550 ± 120 700 ± 80 250 ± 27	0,2 ± 0,2 0,6 ± 0,1 1,3 ± 0,1	$5{\cdot}10^{-3}$

4. Concluding Remarks

Quantitative characteristics of the effect of chlorine-containing pesticides on the structural and functional activity of red blood cells were obtained. From the results presented in the table, it can be seen that the studied chlorine-containing drugs reduce the functional

(membrane) activity of red blood cells and change their mechanical hemolytic resistance to drugs and ultrasound, both individually and in combined action(see table 2.

The data obtained indicate that PCP Na, PCNB, heptachlor and Rohor are relatively weak inhibitors of the enzymatic activity of ACE in erythrocyte membranes, but have a pronounced structural-determinant effect, namely, they can cause hemolysis in an isotonic medium (Na PCP, Rohor, trichlorophon) and accelerate (Na PCP, THC, trichlorophon) or slow down (PCNB, heptachlor) the rate of hemolysis (see table 2). Therefore, quantitative indicators that characterize the ULTRASONIC hemolysis of red blood cells can be used as criteria for evaluating the membranotropic action of pesticides. At the same time, when pesticides and ultrasound are combined, their adsorption on the surface of the erythrocyte membrane is observed, which in turn leads to a slowdown in the hemolytic effect and, in turn, to a change in the qualitative and quantitative composition of membrane lipids [10]. The kinetic analysis method is the most effective for determining the mechanism of action of drugs on red blood cell membranes. This creates prerequisites for the search for new medicines for the human body and animals and the offer of new, more effective drugs for their use in agriculture and medicine.

Заключение

Степень гемолиза и защитное действие некоторых поверхностно-активных веществ коррелируют с составом фосфолипидов в мембране эритроцитов; эти изменения могут также отражать нарушения ультраструктуры компонентов эритроцитов, содержащих гидролитические ферменты.

Получены количественные характеристики влияния хлорсодержащих пестицидов на структурно-функциональную активность эритроцитов. Из представленных результатов видно, что исследуемые хлорсодержащие препараты снижают функциональную (мембранную) активность эритроцитов и изменяют их механическую гемолитическую резистентность к лекарственным препаратам и ультразву, как индивидуально, так и в комбинации.

Полученные данные свидетельствуют о том, что ПХФ Na, ПХНБ, гептахлор и Рогор являются относительно слабыми ингибиторами ферментативной активности АПФ мембран эритроцитов, но обладают выраженным структурно-детерминантным действием, а именно могут вызывать гемолиз в изотонической среде (Na ПХФ, рогор, трихлорофон) и ускорять (NaCl ПХФNa, ТХУ-кислота, трихлорофон) или замедлять (ПХНБ, гептахлор) скорость гемолиза(см. таблицу 2). Поэтому количественные показатели, характеризующие УЗ гемолиз эритроцитов, могут быть использованы в качестве критериев оценки мембранотропного действия пестицидов.В то же время при совместном действии пестицидов и ультразвука наблюдается их адсорбция на поверхности мембраны эритроцитов, что в свою очередь приводит к замедлению гемолитического эффекта и к изменению качественного и количественного состава мембранных липидов[10]. Метод кинетического анализа является наиболее эффективным для определения механизма действия лекарственных средств на мембраны эритроцитов. Это создает предпосылки для поиска новых лекарственных средств для организма человека и животных и предложения новых, более эффективных препаратов для их применения в сельском хозяйстве и медицине.

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Effect of maternal high-fat diet on serum brain-derived neurotrophic factor and behavioral reactions in male offspring of wistar rats

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Abstract

Maternal diet affects the development and health of future offspring. Recent studies indicate that excess fat in the female's diet can influence the structure and function of the brain of her pups. An important role in the maturation of the central nervous system (CNS) and the maintenance of its functioning is assigned to brain-derived neurotrophic factor (BDNF). Changes in BDNF concentration in the brain are associated with the occurrence of various neurobehavioral and mental disorders. In this work, we studied the behavioral reactions and the level of serum BDNF in male Wistar rats whose mothers before, during pregnancy and during lactation consumed food with excess fat in the form of margarine (20% of the total daily calorie content) and in the offspring of females who consumed a standard vivarium diet. As a result, an impairment of spatial learning and memory was revealed, as well as increased anxiety in the Morris Water maze, Elevated Plus maze and Open Field maze in pups whose mothers consumed margarine. These disorders were combined with a reduced level of BDNF in the peripheral blood. The data obtained indicate that consumption of excess fat in the form of margarine by dams before pregnancy, during prenatal and early postnatal period contributes to the formation of neurobehavioral disorders in offspring, which is probably associated with reduced BDNF levels in peripheral blood and, consequently, in the brain.

Аннотация

Характер питания матери оказывает влияние на развитие и состояние здоровья ее будущего потомства. Результаты недавних исследований указывают на то, что избыточное количество жиров в рационе самки способно оказывать воздействие на структуру и функционирование мозга ее детенышей. Важная роль в созревании центральной нервной системы и поддержании ее функционирования отводится мозговому нейротрофическому фактору (BDNF). Изменение его содержания в мозге связывают с возникновением различных нейроповеденческих нарушений и психических расстройств. В данной работе были исследованы особенности поведенческих реакций и содержание BDNF в сыворотке крови самцов крыс линии Вистар, чьи матери до, во время беременности и в период лактации потребляли пищу с избыточным содержанием жиров в виде маргарина (20% от общей суточной калорийности) и у потомства самок, потреблявших стандартный рацион вивария. В результате было выявлено нарушение пространственного обучения и памяти, а также повышение уровня тревожности в тестах «Водный лабиринт Морриса», «Приподнятый крестообразный лабиринт» и «Открытое поле» у детенышей, чьи матери потребляли маргарин. Эти нарушения сочетались у них со сниженным уровнем BDNF в периферической крови. Полученные данные указывают на то, что потребление самками избыточного количества жиров в виде маргарина до беременности, в пренатальный и ранний постнатальный период способствует формированию у ее потомства нейроповеденческих нарушений, что вероятно связано со снижением BDNF в периферической крови, а, следовательно, и уровня его экспрессии в головном мозге.

Keywords: High-fat diet, offspring, BDNF, behavioral reactions

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1. Introduction

Currently, a large amount of data has been compiled, which show a significant role of a mother's ration in the development and health status of the future generation. Excessive fat intake before pregnancy, during prenatal and/or early postnatal period increases a risk of the offspring's obesity, metabolic syndrome and diabetes mellitus [1]. In addition, recent studies have demonstrated that except metabolic and endocrine effects, mother's ration influences the formation and functioning of a future child's nervous system, and, consequently, can contribute to the development of mental and behavioral disorders [2, 3]. So, experiments on animals revealed that offspring of female rats which received food with surplus contents of fat before and after pregnancy had sensory-motor and neurobehavioural disorders, and was also prone to depressive and aggressive behavior [4]. Studies on mice models demonstrated that adult offspring had neurogenesis disturbance, changes in the morphology of neurons in the hippocampus and amygdala, which play a significant role in memory, emotional and cognitive processes [5]. Experiments on non-human primates have shown that the offspring of high-fat diet females exhibits a disorder in the functioning of neurotransmitter systems (serotonin and GABA-ergic). As a result there was an impairment of signaling through synapses in the fetus during its development and an increase in the risk of neuropsychiatric and neurobehavioral disorders [6]. The mechanisms of such deviations remain to be insufficiently explored up to date. Currently, there are several ways to explain the effect of maternal high-fat diet on offspring neurodevelopment: neuroinflammation; increased oxidative stress, dysregulated insulin, glucose, and leptin signaling; impaired serotonergic and dopaminergic signaling; and perturbations in synaptic plasticity [7].

Neurotrophins play a significant role in synapsis functioning and brain development, BDNF being one of the key and most studied of them. In the brain, BDNF is active in the hippocampus, amygdala and cerebral cortex – areas, which are responsible for learning and memory. BDNF plays an important role in the development of the nervous system, neurogenesis, neurons survival and synaptic plasticity, which determines its role in the mechanisms leading to different mental and neurological diseases [8]. Results of some researches show the influence of a high-fat diet on BDNF level and cognitive functions of adults [9, 10, 11, 12, 13]. So, Molteni et al. [11] in experiments on rats consuming excessive saturated fatty acids and refined carbohydrates revealed a decrease in the efficiency of spatial learning, neurogenesis suppression and reduced BDNF level in hippocampus. There was also revealed a decline in synapsin 1 and CREB proteins that are involved in the formation of neuronal plasticity and synaptic transmission, levels of which are controlled by BDNF. Similar results were obtained in the works of Wu et al. [12] who found that a diet high in saturated fat decreases the level of BDNF and proteins regulated by it. This was shown to due to oxidative stress arising under such conditions. Modeling the "western" diet rich in fats and carbohydrates on rats, Stranahan et al. [13] revealed cognitive deterioration, decreased synaptic plasticity and dendritic spines density as well as reduced level of BDNF in the hippocampus. Therefore, it can be assumed that high-fat diet causes synapsis function disorder through the mechanism which includes decreased BDNF level and dendritic spines atrophy.

However, the data regarding the effects of maternal diet on the offspring's brain, neurotrophins levels and cognitive functions are more limited and mixed. Page et al. [14] have shown that consumption of food with high fat content by female rats deteriorates spatial memory of their pups in Morris Water maze test. At the same time, Bilbo and Tsang [15] obtained opposite results. They found improvement of spatial memory of rats, when their mothers' ration contained excessive saturated or trans-fats. Tozuka et al. [16] studied the effect of maternal high-fat diet induced obesity in female mice on the presence of cognitive impairment and the production of BDNF in the hippocampus of their offspring. As a result, spatial memory deterioration was revealed as well as neurogenesis alteration and BDNF level decrease in the hippocampus of pups during the early postnatal period. However, such changes were not observed in the adult offspring of these mice. On the contrary, Rincel et al. [17] described protective effects of a mother's high-fat diet on the nervous system of the offspring. Thus, it was found that excess fat in the female's diet before, during pregnancy and during lactation helps to reduce the effects of early postnatal stress (prolonged separation from the mother) in pups. Offspring revealed normalization of some proteins involved in the development of the nervous system including BDNF as well as reduced anxiety, improved spatial memory and social behavior. In addition, this research did not find changes of BDNF level in the brain of unstressed offspring of mothers consuming high-fat diet. The authors explain this finding by the fact that mother's obesity, rather than a high-fat diet, has an adverse effect on the offspring's brain structure and function.

So, the question about the influence of a mother's diet on the BDNF level and behavioral reactions of its offspring has not been studied completely and requires further research. The literature describes a number of models of high-fat diets with various sources of fat (lard, vegetable oils, margarine) and varying fat percentages from daily calories (from 20 to 60 %) [18, 19, 20]. In the Republic of Belarus, margarine is widely used both in the food industry (baking, confectionery and culinary production) and home cooking. In this regard, the diet with the addition of fat in the form of margarine (20 % of the total daily calories) was used as a model of a high-fat diet for our experiment [19]. Margarine of the selected composition is most widely represented on the Belarusian market.

The aim of this work was to study the effect of maternal

high-fat diet using margarine on behavioral reactions and serum BDNF in male offspring of Wistar rats.

2. Material and Methods

2.1. Animals and diets

All experiments were conducted and approved by the Institute of Physiology of the National Academy of Sciences of Belarus and were in accordance with the guidelines set forth by the European Convention for the Protection of Vertebrate Animals.

The study was conducted on 20 immature female Wistar rats at the age of 1,5 months. The animals were divided into two groups and kept under 12/12-h light-darkness cycle at a temperature of 22 ± 2 °C with free access to water and food (at libitum). The first group (SD, n=10) received a standard vivarium diet. The second group (HFD, n=10) consumed a high-fat diet rich in fats in the form of margarine (20 % of the total daily calories) for 8 weeks. The margarine used in the experiment had the following composition: refined deodorized vegetable oils in natural and modified form (sunflower oil, rapeseed oil, palm oil), water, edible salt, dry whey, sugar, emulsifier: mono- and diglycerides of fatty acids, preservative, dye (beta-carotene) and flavoring. Mass fraction of fat was 82%.

After 8 weeks, female rats in the estrus phase mated with males. Pregnancy was confirmed by the presence of sperm in vaginal smears. During pregnancy and lactation, females from SD group continued to consume the standard diet of vivarium, and HFD females – food with excess fat content. On the 30th day of life, offsprings were separated from their mothers into another cages and divided into two groups depending on the female's diet. The first group (offspring SD, n=19) were males whose mothers were kept on a standard vivarium diet, and the second (offspring HFD, n=27) were males whose mothers consumed a high-fat diet. In the present study, only male offspring were used. Till the end of the experiment, the pups were kept under normal conditions, being on the standard diet of the vivarium, and were removed from the experiment at the age of 3 months by decapitation with prior anesthesia .

2.2. Determination of Visceral Fat Mass

One day after weaning, the female rats were weighed and removed from the experiment by decapitation with prior anesthesia. Visceral fat mass was assessed by weighing the total perirenal and inguinal adipose tissues after dissection.

2.3. Morris Water maze

The Morris Water maze (MWM) consists of a round tank 60 cm in diameter and 40 cm deep, filled with water. The water temperature was 24 ± 2 °C. The tank was divided into four sectors with four equidistant from each other points, marked as North (N), East (E), South (S) and West (W). A circular platform (10 cm in diameter) was submerged in the center of the target sector (South-West sector). The platform remained in the same sector throughout the experiment. High contrast visual signals were placed on the pool wall in each quadrant.

Using the Morris Water maze spatial learning and memory in male offspring of 51-56 days old were tested. The experiment lasted two days [21]. On the first day, the animals were trained using a visible platform. The platform had a bright color and rose 2-3 cm above the surface of water. Each animal was subjected to four tests with an interval of 30 ± 10 min. The rat was carefully placed in the pool water at points N or E between sectors equidistant from the platform (the start of the rat alternated first from point N, then E, then again N and E). The test time was 180 sec to find the visible platform. Animals that could not cope with the task in this period of time were sent to the platform manually. The rats remained on the platform for 10 sec before being removed. The animals were dried with towels and placed in cages. On the second day, a series consisting of three trials was conducted to find the hidden platform. The platform was placed 2 cm below the water surface in the same sector as on the first day of testing. As before, the interval between tests was 30 ± 10 min. The rat was launched from point N, then E, then again N.

In each test, the time from the animal's immersion in water to finding a hidden platform was measured (escape latency time) to assess the development of spatial memory.

2.4. Open Field maze

Open Field maze is a circular arena with a diameter of 120 cm and a wall height of 55 cm, divided into 12 peripheral and 7 central quadrants. In the Open Field test, we studied explorative and motor activity, as well as the level of anxiety in male offspring of 57-62 days old. The test time was 3 min. Before placing the rat, the maze was each time cleaned with 70 % ethanol. The rat was placed in the center of the arena. The latent period of the animal's exit from the center was recorded, the number of line crossings in the periphery and in the center, the quantity of rearings, acts of grooming and droppings (boluses) were counted.

2.5. Elevated Plus maze

Elevated Plus maze is a cross-shaped labyrinth located at a height of 60 cm from the floor. It consists of four opposite sleeves, two open (50 cm x 10 cm) and two closed (50 cm x 10 cm). Closed sleeves are surrounded by walls 40 cm high, open sleeves have no walls. Using this test, we studied explorative and motor activity, as well as the level of anxiety in male offspring of 63-69 days old. Before each rat was placed in the labyrinth, all sleeves were cleaned with 70 % ethanol. The rat was placed in the center of the maze facing the open sleeves. The test duration was 5 min. The results

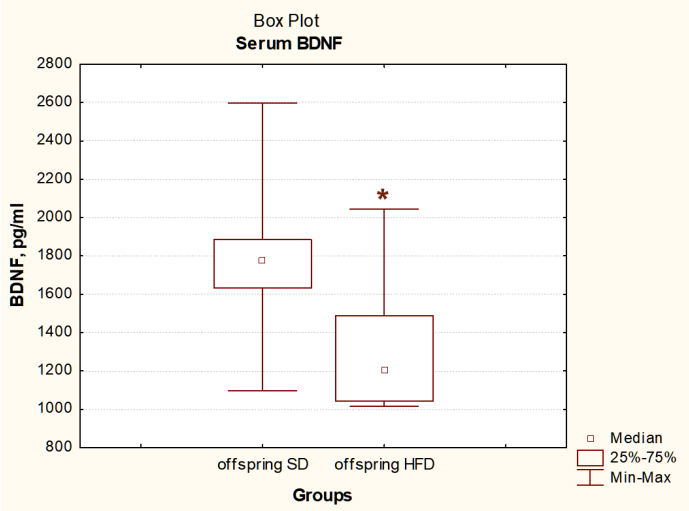

Figure 1 | Serum BDNF in offspring. Values are Median [25%; 75%]. * p ≤ 0,05 offspring HFD versus offspring SD. (Mann–Whitney U test). Уровень сывороточного БДНФ у потомства. Значения представлены в виде медианы [25%; 75%]. * p ≤ 0,05 достоверные отличия группы «Потомство ВЖД» от группы «Потомство СД». (U критерий Манна-Уитни).

were recorded using a Logitech Webcam 905 camera (Logitech, China). Data processing was carried out using the program ANY-Maze (Stoelting Ing., USA).

2.6. BDNF protein level determination

For serum preparation, the blood was collected into sampling tubes and left to coagulate for 1 h. Then it was centrifuged at 2500 g for 10 min to obtain serum. Sera were divided into aliquots and stored at −80°C until BDNF measurements. BDNF were measured by ELISA (FineTest, China) in accordance with the manufacturer's instructions.

2.7. Statistical analysis

Statistical analysis was performed using Statistica 7.0. Normality was defined by the Shapiro-Wilk test. Parametric variables were expressed as means ± standard error, and analyzed by independent t-test. The non-parametric variables were expressed as median, 25 and 75 percentile and analyzed by Mann–Whitney U test. For all statistical tests p≤0,05 was considered significant.

3. Results

The weight of mothers who consumed high-fat diet (margarine) did not differ from the weight of mothers on a standard diet of vivarium (230 ± 10 g and 253 ± 5 g, respectively). The results of weighing visceral adipose tissue (VAT) of female rats from two groups also did not reveal significant differences. So, VAT mass in the HFD group was 3,03 [2,69; 6,67] g, and in the SD group - 2,65 [1,80; 3,20] g. Thus, the diet used didn't lead to the development of obesity in female rats.

According to the results of the experiment in the Morris Water maze, the HFD offspring showed a significant (p ≤ 0,05) increase in the escape latency time in the first two trials (T1, T2) on the second day of the test (24 hours after training using the visible platform). So, the time to reach the platform hidden under water in the T1 test was 10 sec, in the T2 test – 5 sec, and in the control group (offspring SD) – 5 and 4 sec, respectively (Table 1). The data obtained indicate impairment of the spatial learning and memory in offspring HFD.

The Open Field test is designed to assess the behavioral reactions in rodents under stressful conditions that occur in response to placing an animal in open space with intense lighting. The results of our experiment showed an increased anxiety and motor activity in offspring HFD, which revealed a significant (p ≤ 0,05) increase in the number of line crossings in the center and quantity of rearings, as well as a decrease in the number of grooming acts (Table 2).

An increased anxiety in HFD offspring was also confirmed by the Elevated Plus maze test. The method is based on the fact that rodents by their nature stay in secluded places (closed zone) and avoid open spaces. In the conditions of our experiment, HFD offspring showed a passive behavior in the "safe" closed zone: increase in inactivity time, decrease in activity time, decrease in the number of grooming acts and its duration (Table 3).

Concerning the level of serum BDNF, its significant (p ≤ 0,05) decrease compared to control (offspring SD) was found in the offspring of rats whose mothers consumed margarine before and during pregnancy, as well as during lactation (Fig. 1). So, the level of BDNF in the offspring HFD was 1208 [1042; 1489] pg/ml, and in the offspring SD − 1778 [1631; 1887] pg/ml.

4. Discussion

In this study, it was found that the consumption of excess fat in the form of margarine the above-mentioned composition by female Wistar rats before and during pregnancy, and also during the lactation period facilitates the formation of its offsprings's neurobehavioural disorders.

Table 1 | Effect of maternal high-fat diet on offspring's spatial learning and memory in Morris Water maze. Values are Median [25%; 75%]. * p ≤ 0,05 offspring HFD versus offspring SD (Mann–Whitney U test). T1, T2, T3 – series hidden platform trials.
Влияние высокожировой диеты матери на пространственное обучение и память у потомства в тесте «Водный лабиринт Морриса».Значения представлены в виде медианы [25%; 75%]. * p ≤ 0,05 достоверные отличия группы «Потомство ВЖД» от группы «Потомство СД» (U критерий Манна-Уитни). T1, T2, T3 – серии испытаний со скрытой платформой.

Groups	Escape latency time (s)		
	T1	T2	T3
offspring SD	5 [3;12]	4 [2; 6]	4 [2; 9]
offspring HFD	10 [5; 21]*	5 [4; 8]*	4 [3; 8]

So, in the tests Morris Water maze, Elevated Plus maze and Open Field maze an impairment of spatial learning and memory was revealed, as well as an anxiety level increase in rats pups, whose mothers had excessive fat in their diet. These tests are in line with the results obtained by Page et al. [14] and contradict with the data of Bilbo and Tsang [15], which, on the contrary, gave evidence of more successful test performance by pups in the Morris Water maze. It could be due to the fact that Bilbo and Tsang [15] used in their experiment sexually mature female animals which were on a high-fat diet during a shorter period of time (4 weeks before impregnation). Improved spatial memory and a decreased anxiety level in pups of female animals, which were on a high-fat diet, were also observed in the experiments of Rincel et al [17]. But these authors started to use such a ration only from the first day of gestation. Consequently, it can be assumed that not only excessive fat in the ration but also duration of its consumption by a mother before pregnancy influences cognitive functions of its offspring.

As for BDNF level, we revealed its significant decrease in the peripheral blood of the offspring of female rats which consumed a high-fat diet. Today, there are few researches concerning the influence of mother's nutrition on the BDNF level of its young ones and they mainly concern BDNF content in the brain tissues. Concentration of serum BDNF is known to be positively correlated with its content in the brain [8]. Consequently, the results obtained by us can reflect the changes of this neurotrophin in the central nervous system. Our results are in line with the data of Tozuka et al. [16] who revealed a decrease of BDNF in the hippocampus of pups from female rats with obesity provoked by a high-fat diet. At the same time, Rincel et al. [17] did not reveal such changes in their works. They explained it by the fact that a mother's obesity (rather than excessive content of fats in its ration) causes a disturbance of this neurotrophin expression. According to the results of our research, obesity did not develop in female rats. Consequently, a conclusion can be drawn that in our case, a change in the BDNF level is related to the excessive content of fat in a mother's ration. Since this neurotrophin plays an important role in maturation of the central nervous system and maintaining of its functioning, neurobehavioural disorders observed in this experiment can be explained by BDNF level decrease.

Table 2 | Effect Effect of maternal high-fat diet on offspring's behavioural reactions in Open Field maze. Values are Median [25%; 75%]. * p≤0,05 offspring HFD versus offspring SD (Mann–Whitney U test).
Влияние высокожировой диеты матери на поведенческие реакции у потомства в тесте «Открытое поле». Значения представлены в виде медианы [25%; 75%]. * p ≤ 0,05 достоверные отличия группы «Потомство ВЖД» от группы «Потомство СД» (U критерий Манна-Уитни).

Groups	Line crossing: center square	Rearing (act)	Grooming (act)
offspring SD	4 [1;6]	9 [6; 14]	4 [3; 7]
offspring HFD	6 [2; 13]*	16 [9; 23]*	1 [1; 2]*

Table 3 | Effect of maternal high-fat diet on offspring's behavioral reactions in Elevated Plus maze. Values are Median [25%; 75%]. * p ≤ 0,05 offspring HFD versus offspring SD. (Mann–Whitney U test).
Влияние высокожировой диеты матери на поведенческие реакции у потомства в тесте «Приподнятый крестообразный лабиринт». Значения представлены в виде медианы [25%; 75%]. * p ≤ 0,05 достоверные отличия группы «Потомство ВЖД» от группы «Потомство СД». (U критерий Манна-Уитни).

Groups	Time active (s)	Time inactive (s)	Grooming (act)	Time grooming
offspring SD	190 [141; 208]	110 [92;159]	7 [5; 13]	46 [21;80]
offspring HFD	146 [108; 190]*	154 [110; 192]*	4 [4; 7]*	21 [7; 35]*

5. Concluding Remarks

Thus, based on the data obtained, it can be concluded that neurobehavioral disorders (increased anxiety level, impared spatial learning and memory) are observed in male Wistar rats whose mothers consumed excessive fat in the form of margarine before, during pregnancy and during lactation. One of the mechanisms of the formation of such disorders can be related to decreased levels of BDNF protein. In addition, taking into account the results presented in this work, as well as the data of other authors [14, 15, 16, 17], it can be assumed that the duration of a high-fat diet consumed by a mother before pregnancy is also of great importance for the emergence of the nervous system functioning disorders in its offspring.

Заключение

Таким образом, исходя из полученных данных можно сделать вывод, что у крыс мужского пола линии Вистар, матери которых потребляли избыточное количество жиров в виде маргарина до, во время беременности и в период лактации, наблюдаются нейроповеденческие нарушения (повышение уровня тревожности, ухудшение пространственной памяти и способности к обучению). Одним из механизмов формирования таких нарушений может быть снижение уровня белка БДНФ. Кроме того, учитывая результаты, представленные в этой работе, а также данные других авторов [14, 15, 16, 17], можно предположить, что длительность применения высокожировой диеты матерью до беременности также имеет важное значение в возникновении нарушений функционирования нервной системы у ее потомства.

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S-glutathionylation of proteins in various types of neurodegenerative pathology and protective effects of pantothenic acid derivatives

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Abstract

We studied changes in S-glutathionylated proteins (PSSG) content in rat brain structures *in vivo* after administration of Fe+LPS, aluminum chloride, rotenone, and 3-nitropropionic acid, and in vitro experiments with oxidative stress on rat brain tissue culture or on subcellular fractions, as well as possibility of correcting these changes with pantothenic acid precursors – panthenol, pantethine and homopantothenic acid (HPA). We have shown that the content of PSSG significantly increases in brain structures in all the models of neurotoxicosis, and this increase is observed to the greatest extent precisely in those structures (hemispheres, hippocampus, basal ganglia) where a particular neurotoxin has the most pronounced effect. Thus, the protein glutathionylation can initiate dysfunction of proteins and contribute to the development of neurodegeneration. Precursors of CoA reduce S-glutathionylation of proteins, since HPA, which is not a precursor of CoA, does not have a protective effect in relation to PSSG.

Аннотация

Были изучены изменения в содержании S-глутатионилированных белков (PSSG) в структурах мозга крыс *in vivo* после введения Fe + ЛПС, хлорида алюминия, ротенона и 3-нитропропионовой кислоты, а также в экспериментах in vitro при окислительном стрессе на культуре ткани мозга крысы или на субклеточных фракциях, а также возможность коррекции этих изменений с помощью предшественников пантотеновой кислоты – пантенола (ПЛ), пантетина (ПТ) и гомопантотеновой кислоты (ГПК). Установлено, что содержание PSSG значительно увеличивается в структурах головного мозга во всех моделях нейротоксикоза, и это увеличение наблюдается в наибольшей степени именно в тех структурах (больших полушариях, гиппокампе, базальных ганглиях), где данный нейротоксин проявляет наиболее выраженное действие. Очевидно, процесс S-глутатионилирования белков может инициировать нарушения их функций и способствовать развитию нейродегенерации. Предшественники биосинтеза КоА снижают S-глутатионилирование белков, тогда как ГПК, который не является предшественником КоА, не оказывает защитного действия на этот показатель.

Keywords: Protein S-glutathionylation, neurodegenerative pathology, neurotoxicosis, brain structures, panthothenic acid derivates.

1. Introduction

Currently known, disorders in brain structures in such neurodegenerative pathologies as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), etc., are accompanied by the development of oxidative stress [1, 2]. In the mechanisms that ensure the maintenance of redox balance in the brain tissue, the glutathione system plays an important role [3, 4].

The levels of GSH were proposed to use the determination of the GSH level as a marker of moderate kognitive impairment in AD [5].

In addition to participating in redox reactions, glutathione is involved in protein glutathionylation reactions. Protein S-glutathionylation is a specific oxidative post-translational modification characterized by the reversible formation of a mixed disulfide bond between Cys protein residues and glutathione [6]. The high level of GSH in the cells and the easy conversion of sulfenic acids and S-nitro derivatives to

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glutathione-mixed disulfides suggest that reversible glutathionylation may be a common mechanism of redox signal transmission and regulation of the activity of redox-sensitive thiol-containing proteins [7]. Currently, over 100 proteins have been identified, the activity of which changes as a result of post-translational S-glutathionylation [6]. For example, as a result of S-glutathionylation, the activity of apoptosis regulation proteins (caspase-3), folding and degradation proteins (heat shock protein HSP70), energy metabolism proteins (glyceraldehyde-3-phosphate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase complex) significantly changes [8, 9, 10].

Glutathionylation can occur through non-enzymatic or enzymatic reactions. Enzymes that catalyze the reaction of formation of S-glutathionylated proteins (PSSG) are thioltransferases - glutathione-S-transferase P, glutathione-S -transferase O1 of the omega class. The non-enzymatic formation of S-glutathionylated proteins (PSSG) depends on the availability of GSH and / or GSSG. PSSG are easily and reversibly formed via GSH binding by Cys residues in target proteins under the action of glutaredoxins and thioredoxins [4, 13].

S-glutathionylation proceeds both under normal physiological conditions when the GSH / GSSG ratio in the brain is about 100: 1 [14], and in conditions of changes of redox balance in oxidative stress, when the GSH / GSSG ratio can significantly decrease. S-glutathionylation, on the one hand, is a protective mechanism against the action of reactive oxygen species, and, on the other hand, by changing the activity of certain enzymes, it can lead to changes in energy metabolism, folding and protein degradation, and regulation of apoptosis, which play an important role in the pathogenesis of neurodegenerative disorders [1–3, 6, 7]. Recent studies have shown that glutathionylation of specific proteins can contribute to the onset or progression of AD, PD, HD and other neurodegenerative diseases [9-12, 15].

Based on this, we studied changes in the PSSG content in rat brain structures in different experimental models of neurodegenerative diseases, which are characterized by different mechanisms for the development of pathological changes in brain tissue. What was common to them was that in all cases the development of oxidative stress was observed and, accordingly, a shift in the redox balance and changes in the glutathione system [3, 4]. We also studied the possibility of correcting these changes with pantothenic acid derivatives. Earlier, we found the presence of pronounced neuroprotective activity in CoA precursors, carried out through interaction with the glutathione system [16].

2. Material and Methods

Experimental models were performed on male Wistar CRL: (WI) WUBR rats weighing 180–200 g, kept under standard vivarium conditions. All experiments with laboratory animals were carried out in accordance with ethical standards, as well as the rules for conducting scientific work using experimental animals in scientific research, compiled on the basis of recommendations and requirements of the World Animal Welfare Society (WSPA) and the European Convention for the Protection of Experimental Animals (Strasbourg, 1986).

Derivatives of pantothenic acid (PA) D-panthenol (PL) and D-pantethine (PT), which are the precursors of CoA biosynthesis, as well as homopantothenic acid (HPA, hopantene), which is unable to convert to CoA, were used as modulators of metabolic disturbances during oxidative stress. These drugs were administered at a dose of 200 mg / kg, intragastrically.

Administration of iron (II) gluconate and E. coli lipopolysaccharide (LPS) to model PKAN (pantothenate kinase-associated neurodegeneration) [17, 18]. 10–12-Day-old rats weighing 20 ± 5 g were injected with iron (II) gluconate (30 mg / kg, intragastrically) for 20 days. From 21 days, PA derivatives were administered for 14 days. The day before decapitation, E. coli lipopolysaccharide (200 μg / kg, ip) was administered.

Administration of aluminum chloride for modeling AD [19]. Aluminum chloride (200 mg / kg, intragastrically) was administered daily to rats for 6 weeks. From the 5th week of the experiment, PA derivatives were administered daily for 14 days.

Administration of rotenone for modeling PD [20, 21]. Rats were injected with rotenone daily for 6 weeks (2.5 mg / kg, subcutaneously, diluent — a mixture of DMSO with sunflower oil). From the 5th week of the experiment, PA derivatives were administered daily for 14 days.

Administration of 3-nitropropionic acid for modeling HD [22]. Rats were administered 3-nitropropionic acid daily for 14 days (NPA, 10 mg / kg, intraperitoneally). PA derivatives were administered daily for 14 days, too.

Brain structures examined. We studied the level of PSSG in the cerebral hemispheres, hippocampus, basal ganglia, brain stem and cerebellum isolated from rat brain, as well as in brain cell culture from rat embryos.

Primary cell culture and isolation subcellular fractions from brain tissue.

The primary brain cell culture of 18-day-old rat embryos was isolated using mechanical and enzymatic tissue disaggregation with trypsin [23, 24]. Cells were incubated in Eagle's medium (MEM) containing 5% thermally inactivated rat serum, 5.55 mM glucose, 2 mM glutamine, 20 U / ml penicillin and 20 U / ml streptomycin in 50 mm diameter plastic Petri dishes pretreated poly-D / L-lysine [23, 24]. The cell culture was incubated at 37 ° C in a gas mixture containing 5% CO2. Cell viability in culture was assessed by the release of lactate dehydrogenase into the extracellular medium.

In the another experiment subcellular fractions were isolated from rat cerebral tissue by differential centrifugation using a selection medium containing 0.32 M sucrose, 10 mM Tris HCl, pH 7.4 and 1 mM EDTA. Mitochondrial sediment was resuspended in isolation medium at the rate of 0.4 ml of

Table 1 | The effect of PL and PT on the content of PSSG (μmol / g tissue) in the brain structures after the administration of iron (II) gluconate and LPS, (M ± SEM, n = 8). Влияние ПЛ и ПТ на содержание PSSG (мкмоль / г ткани) в структурах мозга крыс после введения глюконата железа (II) и ЛПС (M±SEM, n=8). Notes: * - p <0.05 as compared to the control group, # - p <0.05 as compared to the Fe+LPS group.

Groups	Hemispheres	Basal ganglia	Hippocampus
Control	0.1 ± 0.1	0.1 ± 0.03	0.11 ± 0.04
Fe+LPS	0.13 ± 0.03*	0.11 ± 0.01*	0.12 ± 0.01*
Fe+LPS+PL	0.13 ± 0.01*	0.08 ± 0.02*#	0.11 ± 0.02#
Fe+LPS+PT	0.12 ± 0.01*#	0.11 ± 0.01*	0.09 ± 0.03*#

medium per mitochondrial precipitate isolated from 1 g of tissue. Cells and mitochondria were destroyed using a lysis buffer containing 20 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100.

The content of PSSG was determined by spectrofluorimetric method using 2,3-naphthalenedicarboxaldehyde (NDA) by the technique of Menon, Board [25] with our modifications. In order to avoid formation of free thiols (predominantly GSH) in the samples, they are blocked by the inclusion of 10 mM NEM (N-ethylmaleimide) in the homogenizing solution. After centrifugation a tissue extract was precipitated with twice the volume of ice-cold acetone to remove the alkylated cellular free thiols and excess NEM. After second centrifugation, the supernatant was discarded and the precipitated proteins were washed again in ice-cold acetone and allowed to air dry. The pellet was suspended in 0.5 M Tris·HCl, pH 8.2, containing 0.1% Triton X-100.

The reducing agent tris(2-carboxyethyl)phosphine (TCEP) was added to the protein solution (final concentration of 5 mM) to break the disulfide protein bonds and elute the protein-bound GSH. The deglutathionylated proteins was then precipitated by the addition 5-sulfosalicylic acid on ice for 30 min, and the precipitated proteins were removed by centrifugation. Releasing GSH from proteins in the supernatant was detected with NDA spectrofluorimetrically (Ex=485 nm, Em =520 nm). The results were calculated as nmol GSH/mg protein by reference to a standard curve generated with GSH.

Total protein was determined by the Bradford method [26].

Statistical processing of experimental data was performed using Microsoft Excel 2016, GraphPad Prism 6.0. The experimental data were presented as M ± SEM, where M is the average value, SEM is the standard error of the mean. The significance of intergroup differences was evaluated using one-way analysis of variance (ANOVA) using the Tukey test. In all cases, differences were considered statistically significant at a value of p <0.05.

3. Results

We have studied changes in the content of PSSG in rat brain structures in different models of neurodegeneration.

Since iron does not penetrate well through the blood-brain barrier in adult animals, we administered iron preparations to 10–12-day-old rat pups when the barrier permeability is still high [17, 18]. This contributes to the accumulation of iron in the brain tissue, and mainly in the striatum of the cerebral hemispheres. An additional single administration of LPS to animals on the 34th day of their life leads to the development of inflammation and can serve as an experimental model of PKAN, which is characterized by the accumulation of iron and due to impaired CoA biosynthesis due to a genetic defect in the key enzyme of its biosynthesis – pantothenate kinase [27]. The control group consisted of animals of the same age, which were injected with a solution of sodium chloride 0.85 %.

We found that the content of PSSG after administration of iron (II) gluconate and LPS in the cerebral hemispheres increased by 26%, in basal ganglia by 11% and in the hippocampus by 14% (table 1).

Although PL did not affect the process of S-glutathionylation of proteins in the cerebral hemispheres, it was shown that it decreased S-glutathionylated proteins in the hippocampus to the control level, and even lower than the values in the control in basal ganglia (p <0.05). Against this background, PL did not affect the process of S-glutathionylation of proteins in the cerebral hemispheres, reduced their content in the hippocampus to the control level, and in basal ganglia reduced their content even lower than the values in the control (p<0.05). Conversely, tThe presence of PT did not affect this indicator of basal ganglia, decreased the level of PSSG in hemispheres, and in hippocampus decreased it below the control values.

To model AD symptoms in rats, we used a model with intragastrically administeredration of aluminum chloride for 6 weeks. This led to the development of neurotoxicosis in animals, accompanied by disturbances in the redox balance and pronounced changes in the redox potential of the glutathione system in brain tissue [28].

The action of aluminum chloride led to a significant increase in the content of PSSG in all the rat brain structures we studied, which, obviously, is an indicator of an increase in the post-translational modification of proteins under conditions of a shift in the thiol disulfide balance (table 2). It can be noted that the level of PSSG changes the least in the brain stem. The introduction of all PA derivatives (PL, PT, and HPA) led to the return of restored the PSSG content in hemispheres and hippocampus to the control level, and even lower than the control values in other brain structures.

Together, these results indicate Thus, that the process of S -glutathionylation of proteins under the action of aluminum chloride is activated in all brain structures of rats, and PA derivatives are effective correctors that reduce post-translational modification of proteins under conditions of

Table 2 | The effect of PL, PT, and HPA on the content of PSSG (nmol / mg protein) in the brain structures after the administration of AlCl3 (M±SEM, n=8). Влияние ПЛ, ПТ и ГПК на содержание PSSG (нмоль / мг белка) в структурах мозга крыс после введения AlCl3 (M±SEM, n=8). Notes: * - p <0.05 as compared to the control group, # - p <0.05 as compared to the AlCl3 group

Groups	Hemispheres	Basal ganglia	Hippocampus	Brain stem	Cerebellum
Control	0.47 ± 0.04	0.52 ± 0.04	0.25 ± 0.04	0.28 ± 0.03	0.24 ± 0.03
$AlCl_3$	0.60 ± 0.05*	0.58 ± 0.02*	0.28 ± 0.05*	0.31 ± 0.05*	0.29 ± 0.05*
$AlCl_3$ +PL	0.46 ± 0.08#	0.48 ± 0.05#	0.26 ± 0.04#	0.27 ± 0.03#	0.23 ± 0.03#
$AlCl_3$ + PT	0.47 ± 0.06#	0.40 ± 0.06*#	0.24 ± 0.02#	0.26 ± 0.02#	0.21 ± 0.02#
$AlCl_3$ + HPA	0.34 ± 0.02*#	0.51 ± 0.06#	0.25 ± 0.06#	0.24 ± 0.04#	0.24 ± 0.04#

oxidative stress in the central nervous system.

Rotenone is one of the neurotoxins used to model PD *in vitro* and *in vivo* [21]. Administration of rotenone to animals causes biochemical, histological and behavioral symptoms similar to those observed in patients with PD. Rotenone is an inhibitor of the mitochondrial I complex I of the electron transport chain of mitochondria, resulting in the formation of free radicals and the development of oxidative stress [229]. In our experiments, it was found that the effect of rotenone was accompanied by an increase in the content of PSSG in the cerebral hemispheres by 20%, the hippocampus by 18% and, most notably, in the basal ganglia by 56 % especially pronounced (by 56%) in the basal ganglia (table 3). Predictably, rotenone is known to cause the most prominent damage to neurones in the basal ganglia. It is well known, that in this structure of the brain rotenone causes the most pronounced damage to neurons.

PL and PT contributed to the weakening of the effect of rotenone on this indicator in the cerebral hemispheres and the hippocampus, returning it to the values in the control, while in basal ganglia the effect of their exposure was insufficient to return the PSSG level to normal. HPA had no effect on the content of PSSG against rotenone.

Table 3 | The effect of PL, PT, and HPA on the content of PSSG (nmol / mg protein) in the brain structures after the administration of rotenone (M±SEM, n=8). Влияние ПЛ, ПТ и ГПК на содержание PSSG (нмоль / мг белка) нмоль / мг белка) в структурах мозга крыс после введения ротенона (M±SEM, n=8). Notes: * - p <0.05 as compared to the control group, # - p <0.05 as compared to the rotenone group .

Groups	Hemispheres	Basal ganglia	Hippocampus
Control	0.50 ± 0.02	0.57 ± 0.02	0.51 ± 0.03
Rotenone	0.59 ± 0.02*	0.88 ± 0.03*	0.60 ± 0.02*
Rotenone +PL	0.52 ± 0.02#	0.72 ± 0.02*#	0.52 ± 0.01#
Rotenone +PT	0.51 ± 0.02#	0.69 ± 0.02*#	0.51 ± 0.01#
Rotenone +HPA	0.53 ± 0.01*	0.90 ± 0.03*#	0.61 ± 0.02*

NPA is a mitochondrial toxin that causes selective degeneration of neurons in the striatum and the development of symptoms characteristic of HD in experimental animals [2930]. Oxidative stress is one of the important factors in the pathogenesis of HD [3031]. In this model, we also observed an increase in the PSSG content in hemispheres by 20% and hippocampus by 23%, while in basal ganglia this increased by 65% (table 4).

PL and PT also returned the PSSG level to control in hemispheres and hippocampus, while in basal ganglia they contributed only to its slight decrease relative to the value against the background of NPA. The effect of HPA was weaker in all studied brain structures.

In order to clarify the mechanisms of the protective effect of PA derivatives on protein glutathionylation processes, we studied changes in this parameter in *in vitro* models of oxidative stress. For this, we used the primary brain cell culture of 18-day-old rat embryos. Derivatives of PA with final concentrations of 10, 25, 50, 100, and 500 μM were added to the cell suspension (0.5–1.0 mg / ml total protein) and preincubation was performed for 30 min at 37 ° C. To induce oxidative stress, 50 μM tBHP was added and samples were incubated for 30 min at 37 ° C. It was shown that the development of oxidative stress initiated by tert-butyl hydroperoxide (tBHP) was accompanied by an almost 3-fold increase in the content of PSSG in the culture of brain cells of rat embryos (figure). The introduction of both PL and PT

Table 4 | The effect of PL, PT, and HPA on the content of PSSG (nmol / mg protein) in the brain structures after the administration of NPA (M±SEM, n=8). Влияние ПЛ, ПТ и ГПК на содержание PSSG (нмоль / мг белка) в структурах мозга крыс после введения 3-нитропропионовой кислоты (M±SEM, n=8). Notes: * - p <0.05 as compared to the control group, # - p <0.05 as compared to the NPA group.

Groups	Hemispheres	Basal ganglia	Hippocampus
Control	0.48 ± 0.01	0.57 ± 0.01	0.53 ± 0.03
NPA	0.58 ± 0.01*	0.93 ± 0.01*	0.65 ± 0.02*
NPA+PL	0.52 ± 0.01#	0.71 ± 0.01*#	0.55 ± 0.01#
NPA+PT	0.53 ± 0.01#	0.69 ± 0.01*#	0.57 ± 0.01#
NPA+HPA	0.51 ± 0.01*	0.83 ± 0.02*#	0.60 ± 0.02*

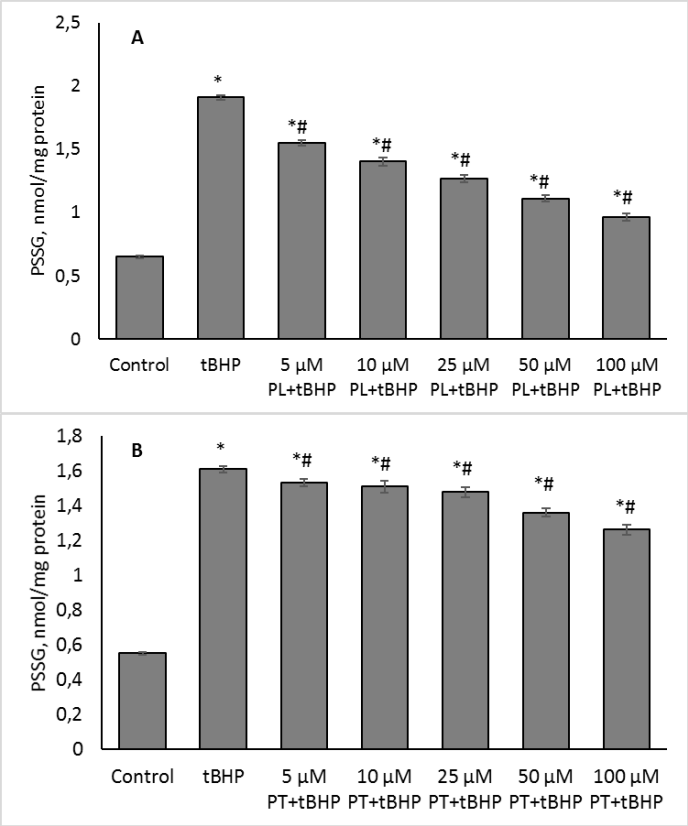

Figure 1 | Influence of tBHP (50 μM), PL (histogram A) and PT (histogram B) on the level of PSSG in the primary brain cell culture of 18-day rat embryos. Влияние tBHP (50 μM), ПЛ (гистограмма А) и ПТ (гистограмма В) на содержание PSSG в первичной культуре клеток головного мозга 18-дневных эмбрионов крыс. Notes: * - p <0.05 as compared to the control group, # - p <0.05 as compared to the tBHP group.

into the incubation medium in a dose-dependent manner contributed to a decrease in the content of PSSG, which indicates a direct effect of the drugs on the processes of glutathionylation of proteins.

In the next experiment, we examined in which subcellular fraction the proteins undergo had the greatest modification of proteins. For this, PL or PT with the final concentrations of 10, 25, 50, 100, and 500 μM were added to the homogenate of brain tissue or a suspension of subcellular fractions (1.5–2 mg / ml of total protein) and preincubated for 30 min at 37 ° C. 100 μM tBHP was added into the samples to induce oxidative stress and incubated during 15 min in 37 °C.

It was established that, against the background of tBHP, the content of PSSG increased both in the total homogenate and in mitochondria, microsomes, and in the cytosol (table 5). In the presence of PL in the incubation medium, the effect of tBHP was not manifested, and the level of PSSG did not differ from the values in the control group either in the total homogenate or in the subcellular fractions studied. However, in the nuclear fraction, the content of S□glutathionylated proteins did not change either against tBHP or in the presence of PL.

4. Concluding Remarks

Protein S-glutathionylation has the ability to regulate a number of biological functions of proteins in the cell, both in normal and pathological conditions. It is generally accepted that S-glutathionylation of proteins is considered one of the mechanisms of protein protection against the oxidative effects of reactive oxygen species [1–3, 6]. We have shown that under the conditions of the development of oxidative stress, the content of PSSG significantly increases in brain structures in all the models of neurotoxicosis that we studied, and this increase is observed to the greatest extent precisely in those structures where a particular neurotoxin has the most pronounced effect. As a rule, with neurodegenerative diseases, pathological changes occur primarily in the cortical and subcortical structures of the hemispheres [32, 33, 34]. According to our data, changes in PSSG levels in the brain stem were minimal in the models of experimental neurodegeneration.

PA derivatives, precursors of CoA, reduce S-glutathionylation of proteins, which can be regarded as a protective effect against protein conformation disorders and preservation of their biological activity during oxidative stress, and these effects are obviously mediated through the CoA system, since HPA, which is not a precursor of CoA, does not have a protective effect in relation to PSSG.

Thus, the activation of S-glutathionylation of proteins is a characteristic sign of metabolic disorders of redox balace in brain tissue during oxidative stress and is observed in different ways of modeling neurodegenerative processes. Given the fact that glutathionylation of proteins can also initiate misfolding of proteins and their aggregation into insoluble complexes, impaired mitochondrial functions, iron accumulation in brain structures, and contribute to the progression of death of neurons, therefore it can be assumed that glutathionylation of proteins can not only protect them from irreversible damage by free radicals during oxidative

Table 5 | The effect of PL (100 μM) on the content of PSSG (nmol / mg protein) in the subcellular fractions of hemispheres in the presence of tBHP (100 μM) (M±SEM, n=4). Влияние ПЛ (100 μM) на содержание PSSG (нмоль / мг белка) в субклеточных фракциях больших полушарий мозга в присутствии tBHP (100 μM) (M±SEM, n=4). Notes: * - p <0.05 as compared to the control group, # - p <0.05 as compared to the tBHP group.

Subcellular fractions	Control	tBHP	tBHP + 0.1 мМ PL
Homogenate 1:10	1.73 ± 0.01	2.31 ± 0.03*	1.81 ± 0.02#
Nucleus	0.14 ± 0.01	0.12 ± 0.01	0.11 ± 0.01
Mitochondria	0.55 ± 0.02	0.92 ± 0.02*	0.63 ± 0.01#
Microsomes	0.28 ± 0.01	0.39 ± 0.01*	0.31 ± 0.02#
Cytosol	0.76 ± 0.02	0.88 ± 0.02*	0.76 ± 0.02#

stress, but also perform certain regulatory functions in the cell, having a close relationship with to CoAlation functions. Recently it has been shown that protein CoAlation is a reversible post-translational modification induced by oxidizing agents and metabolic stress in prokaryotic and eukaryotic cells [335]. During physiological conditions CoA produces metabolically-active derivatives, but may act as an antioxidant in response to oxidative or metabolic stress. The close relationship between CoA-lation and S-glutathionylation is evidenced by the pronounced effects of CoA biosynthesis precursors - PL and PT, but not HPA, on the level of S-glutathionylated proteins in brain structures in the experimental models of neurotoxicosis. It must be taken into account when considering the mechanisms of the pathogenesis of neurodegenerative diseases and may be the rationale for the use of pantothenic acid derivatives for treatment of neurodegenerative disorders.

Заключение

S-глутатионилирование белков обладает способностью регулировать ряд биологических функций белков в клетке как при нормальных, так и при патологических состояниях. Известно, что S-глутатионилирование белков считается одним из механизмов защиты белков от окислительного воздействия активных форм кислорода. Мы показали, что в условиях развития окислительного стресса содержание PSSG значительно возрастает в структурах головного мозга во всех изученных нами моделях нейротоксикоза, и это увеличение наблюдается в наибольшей степени именно в тех структурах, где конкретный нейротоксин обладает наиболее выраженным действием. Как правило, при нейродегенеративных заболеваниях патологические изменения происходят в первую очередь в корковых и подкорковых структурах больших полушарий мозга. В изученных нами моделях экспериментальной нейродегенерации изменения уровня PSSG в стволе головного мозга были наименее выраженными.

Производные пантотеновой кислоты, предшественники КоА, снижают S-глутатионилирование белков, что можно рассматривать как защитный эффект против нарушений конформации белка и сохранение их биологической активности во время окислительного стресса, и эти эффекты, очевидно, опосредуются через систему КоА, поскольку гомопантотеновая кислота, которая не является предшественником КоА, не оказывает защитного действия на этот показатель.

Таким образом, активация S-глутатионилирования белков является характерным признаком нарушений окислительно-восстановительного баланса в ткани мозга при окислительном стрессе и наблюдается при разных способах моделирования нейродегенеративных процессов. Учитывая тот факт, что глутатионилирование белков также может инициировать нарушения сворачивания белков и их агрегацию в нерастворимые комплексы, нарушение функций митохондрий, накопление железа в структурах головного мозга и способствовать прогрессированию гибели нейронов, можно полагать, что глутатионилирование белков может не только защищать их от необратимого повреждения свободными радикалами во время окислительного стресса, но также выполнять определенные регуляторные функции в клетке, тесно связанные с функциями КоА-лирования. Недавно было показано, что КоА-лирование белка является обратимой посттрансляционной модификацией, вызванной окислителями и метаболическим стрессом в прокариотических и эукариотических клетках. В физиологических условиях КоА способствует образованию метаболически активных производных, но он может также действовать как антиоксидант в ответ на окислительный или метаболический стресс. О тесной взаимосвязи между КоА-лированием и S-глутатионилированием свидетельствуют выраженные эффекты предшественников биосинтеза КоА – ПЛ и ПТ, но не ГПК, на уровень S-глутатионилированных белков в структурах головного мозга в экспериментальных моделях нейротоксикоза. Эти факты необходимо учитывать при рассмотрении механизмов патогенеза нейродегенеративных заболеваний и могут служить обоснованием использования производных пантотеновой кислоты для лечения нейродегенеративных заболеваний.

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Biochemical adaptive plant response of corn lines with diferente drought tolerance

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ABSTRACT

The paper deals with impact of water deficit and heat shock on activity of sucrose-phosphate synthase (SPS) – (UDP-glucose: D-fructose-6-phosphat-2-α-glucosyltransferase, E.C. 2.4.1.14) – an enzyme involved in sucrose synthesis, on activity of soluble lectins, as well as sucrose and abscisic acid (ABA) content in germinating kernels of corn lines with different drought tolerance (Zea mays L.). The paper demonstrates that drought-tolerant corn lines are characterized by increased activity of soluble lectins, sucrose-phosphate synthase, abscisic acid and sucrose content in tissues of sprouts under the impact of adverse abiotic factors (water deficit and hyperthermia) compared to reference value.

Аннотация

Изучено влияние водного дефицита и теплового шока на активность сахарозофосфатсинтазы (СФС) - (УДФ-глюкоза D-фруктозо-6-фосфат-2-α-глюкозилтрансфераза, К.Ф. 2.4.1.14) - энзима синтеза сахарозы, активность лектинов, а также содержание сахарозы и АБК в прорастающих зерновках контрастных по признаку засухоустойчивости линий кукурузы (ZeamaysL.). Показано, что pfce [jecnjqxbdst линии кукурузы характеризуются повышением активности растворимых лектинов, сахарозофосфатсинтазы и содержания абсцизовой кислоты сахарозы в тканях проростков при воздействии абиотических неблагоприятных факторов (водного дефицита и гипертермии).

Keywords: *Zea mays L.*, soluble lectins, abscisic acid (ABA), sucrose-phosphate synthase (SPS), sucrose, water deficit, heat shock.

1. Introduction

One of the most pressing issues nowadays is a study of the plants' response to changes in meteorological conditions of environment. This problem is studied now by a new science – meteomics. Advantages of assessment of joint impact of meteorological factors on plants lie in the fact that such approach enables to simulate a real situation in nature.

Drought and high temperature are one of the key factors of the environment limiting crop capacity of grains. Response of the plants to drought and high temperature is very complex and includes interaction between various molecular, physiological and biochemical processes. Significant changes take place in hormone balance of plant cells under various stresses, contributing to change in structure and functions of plant cells under normal conditions into those under stress conditions. Abscisic acid (ABA) plays a key role in regulation of changes in gene expression of plant cells under stress. Under such conditions ABA level rises drastically, which leads to decreasing activity of metabolic processes in cells, namely, total protein synthesis, on the one hand, and induced creation of over a dozen of stress proteins, on the other hand [1, 2, 3].

Synthesis of a number of proteins present under normal conditions, including lectin, increases along with synthesis of stress proteins under adverse conditions, as well as treatment with exogeneous ABA [4, 5, 6]. This is supported by data concerning significant accumulation of lectin in roots of wheat sprouts under osmotic shock and drought, in sprouts in response to salinity of the environment, in cell culture under heat shock, as well as developing wheat kernels under water deficit [7, 8, 9]. Taking into account

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variety of lectins, their presence in various types of plants, and presence of various agglutinating proteins in organs of one and the same plant, one can assume that these molecules play an important physiological role in metabolism. One of the defence mechanisms of lectins under stresses of various nature is their possible impact on cytoskeleton's destabilization and stabilization cycle, which plays a key part in regulation of the plant's response to biotic and abiotic stimuli. Growth in lectin content may also lead to oxidative stress inhibition and decrease in active forms of oxygen, which along with stabilization of the cytoskeleton's new configuration takes the cell back to unexcited state. Based on the given data, lectin may be viewed as a party to nonspecific responses of the plants.

It is known that stress factors (drought, hypothermia, hyperthermia and others) may trigger both decrease and increase of soluble sugar content in cells. One of the most important osmoprotectors under stress conditions is sucrose. It is the main form of sugar transport in most plants. Moreover, sucrose serves as a sign for activation or repression of specific genes in various tissues [10, 11]. Since sucrose content in plants is determined by dynamic balance of its synthesis and hydrolysis, stress-induced change in sucrose content can be contributed to regulation of activity of enzymes synthesizing and engaging this disaccharide into metabolism. Sucrose-phosphate synthase (SPS – E.C. 2.4.1.13) is a key enzyme in sucrose biosynthesis. Major part of the SPS in sucrose accumulation is verified by genetic engineering methods [12]. SPS activity regulation under stress may be affected by metabolite accumulation level, genotypic features, plant's tolerance to an adverse factor [13, 14].

In consideration of the foregoing premises the goal of our study is to identify the patterns and characteristics of changes in the activity of soluble lectins, sucrose phosphate synthase, and the content of sucrose and abscisic acid in maize seedlings with different drought tolerance under water and heat stress in order to create new biochemical methods for assessing drought tolerance.

2. Material and Methods

Three-day young sprouts of corn lines (Zea mays L.) with different levels of drought tolerance were used in the research: drought-tolerant lines Od 329, IK107 zM, non-drought-tolerant lines GK 26, IK107VS3 / 66. Material was prepared and provided by A. O. Belousov, Dr. Sc. (Biology), Head of Laboratory of Genetic and Biotechnological Methods of Corn Selection of the Genetic Selection Institute – National Centre for Study of Seeds and Varieties. Undamaged corn kernels were used in the research. They were let germinate on filter paper in a germination chamber at 25o C at relative humidity 60%. Water deficit (WD) was created by placing the sprouts in the chamber with relative humidity 35-40%. Heat shock (HS) was created by placing the sprouts in the germination chamber at 37o C. Stress factors were applied for 6 hours. Plants in the reference group were subject to optimum humidity conditions at 25o C. Upon completion of exposure dissected aboveground parts of sprouts, endosperm and roots were frozen at -40o C. Lectin activity was determined on the basis of their ability to agglutinate trypsinized erythrocytes of white rats [15]. Total protein content in the extract was determined by Lowry protein assay [16]. Abscisic acid content was determined by gas chromatography [17]. Sucrose content was determined by gas chromatography [18]. Activity of sucrose-phosphate synthase (SPS) – (UDP-glucose: D-fructose-6-phosphat-2-α-glucosyltransferase, E.C. 2.4.1.14) was determined by the standard method [14]. Results were subject to mathematical and statistical processing in accordance with standard practices [18]. Data were expressed as means ± standard deviation (SD) for triple determinations.

3. Results

The following was found in the course of study of the

Table 1 | Induced Change of Activity Soluble Lectins in Sprouts of Corn Lines with Different Levels of Drought Tolerance. Note: * Probable difference P ≤ 0.05 against reference value. Индуцированное изменение активности растворимых лектинов в проростках линий кукурузы с различным уровнем засухоустойчивости. Примечание. * Отличия достоверны при p ≤ 0,05 относительно контроля.

Line	Lectin activity (μg protein/ml)$^{-1}$				% of reference value		
	Reference value	Water deficit	Heat shock	Water deficit + heat shock	Water deficit	Heat shock	Water deficit + heat shock
Drought-tolerant lines							
IK107 zM	14 ± 1	13 ± 1	38 ± 1*	34 ± 3*	97.4	276.0	244.1
Od329	16 ± 2	19 ± 1*	45 ± 2*	26 ± 1*	121.1	281.0	163.0
Non-drought-tolerant lines							
GK26 zM	6.0 ± 0.4	4.6 ± 0.2*	2.4 ± 0.1*	2.2 ± 0.2*	76.2	39.8	37.2
IK107VS$_3$/66	5.3 ± 0.1	3.5 ± 0.1	5.9 ± 0.3	2.5 ± 0.1	66.8	111.4	47.5

Table 1 | IAbscisic Acid Content in Corn Sprouts under Abiotic Stresses (mg/g). Note: * Probable difference P ≤ 0.05 against reference value. Содержание абсцизовой кислоты в проростках кукурузы при абиотических стрессах (мг / г.) Примечание. * Отличия достоверны при p ≤ 0,05 относительно контроля.

Lines	Reference value		Water deficit				Heat shock				Water deficit + heat shock			
	Above-ground part of sprout	Root	Aboveground part of sprout		Root		Aboveground part of sprout		Root		Aboveground part of sprout		Root	
	mg/g		mg/g	% of reference value	mg/g	% of reference value	mg/g	% of reference value	mg/g	% of reference value	mg/g	% of reference value	mg/g	% of reference value
Drought-tolerant lines														
IK107 zM	20 ± 2	9 ± 1	36 ± 3*	181.8	28 ± 2*	320.0	32 ± 3*	160.3	19 ± 1*	220	28 ± 1*	140.3	18 ± 1*	200.0
Od329	8 ± 1	10 ± 1	12 ± 1*	150.7	16 ± 1*	165.7	11 ± 1*	135.6	12 ± 1*	120.0	11 ± 1*	142.8	12 ± 1*	115.0
Non-drought-tolerant lines														
GK26 zM	17 ± 1	7.0 ± 0.4	9 ± 1*	58.8	4.0 ± 0.2*	54.3	14 ± 1	85.2	7.0 ± 0.4	98.2	12 ± 1*	75.0	6.0 ± 0.3*	88.8
IK107VS$_3$/66	5.0 ± 0.2	5.0 ± 0.3	4.7 ± 0.1	95.8	5.0 ± 0.2	99.6	5.0 ± 0.2	105.0	3.0 ± 0.1*	55.5	4.0 ± 0.2	95.0	4.0 ± 0.1	90.0

impact of stress factors, water deficit and high temperature on the total soluble lectin activity in tissues of whole sprouts and ABA content in tissues of the aboveground part and roots of 3-day young sprouts of corn lines with different levels of drought tolerance. The study enabled us to identify the increase in soluble lectin activity (160 – 280 % of reference value) and ABA content (135 – 320 % of reference value) under the given stress factors in drought-tolerant lines, and the decrease in soluble lectin activity (37 - 76 % of reference value) and ABA content (88 – 54,3 % of reference value, GK26zM) under the given stress factors in drought-tolerant lines (Tables 1, 2). Diverse changes in lectin activity under stress factors in plants with various levels of tolerance may occur due to synthesis of isoforms more adjusted to stresses, prevailing synthesis of which facilitates maintaining cellular metabolism at the necessary level. Moreover, reserve forms of lectin mRNA were found through inhibition assay when studying synthesis and accumulation of lectins in cells of the wheat germ [20]. The authors of the paper suggested that there was some kind of a pool of untranslated lectin mRNA and its precursors in wheat cells, and that a stress factor might contribute to mobilization of reserve forms of lectin mRNA into translation stage and trigger acceleration in processing of lectin precursors. It is probable that changes in lectin activity that we observed in tissues of the corn in response to the given stress factors are identified using the same or similar mechanism. One can assume that high level of induced accumulation of lectins in the aboveground part and roots of sprouts of drought-tolerant corn lines under stress factors may be connected with a higher speed of mobilization of reserve forms of lectin mRNA into translation stage, and, consequently, acceleration in processing of lectin precursors. Undoubtedly, this assumption requires verification by experiment and is a task of our further research. Transfer of plants into the state of nonspecific resistance, as well as many other processes connected with impact of stresses, frequently correlate with immunologically significant shifts in the plant's hormone system. In the first place it applies to abscisic acid (ABA), which is called a stress phytohormone. According to reference sources, increase in ABA content preceded increase in lectine content under stress factors of varying nature. Data on ABA and lectin concentration kinetics enabled us to conclude that there is a cause-effect relationship between stress-induced accumulation of ABA and increase in lectin content (activity). However, lectin activity and ABA content may be affected by other factors as well, first of all, genetic nature of plant lines and initial content of lectins and abscisic acid.

According to reference sources, sucrose-phosphate synthase activity and sucrose content may serve as auxiliary criteria for assessment of the plants' response to drought

Table 3 | Sucrose-Phosphate Synthase (SPS) Activity and Sucrose Content in Corn Sprouts Grown amid Water Deficit and Hyperthermia Note: * Probable difference P ≤ 0.05 against reference value. Активность сахарозофосфатсинтазы (СФС) и содержание сахарозы в проростках кукурузы, выращенных в условиях водного дефицита и гипертермии Примечание. * Примечание. * Отличия достоверны при p ≤ 0,05 относительно контроля.

Line	Sucrose-phosphate synthase activity(sucrose μmol / protein mg per hour)			Sucrose content (mg/g on dry basis)		
	Reference value	Water deficit + heat shock	% of reference value	Reference value	Water deficit + heat shock	% of reference value
Drought-tolerant lines						
Od 329						
Aboveground part	3.4 ± 0.1	4.8 ± 0.1*	143.5	15 ± 1	32 ± 3*	215.0
Roots	3.1 ± 0.1	6.7 ± 0.3*	217.2	11 ± 1	25 ± 1*	225.2
IK107 zM						
Aboveground part	3.8 ± 0.1	4.1 ± 0.1	110.0	19 ± 1	28 ± 2*	143.3
Roots	2.9 ± 0.1	3.3 ± 0.1	114.3	15 ± 1	21 ± 1*	139.2
Non-drought-tolerant lines						
GK26 zM						
Aboveground part	3.3 ± 0.1	2.56 ± 0.03*	76.6	14.0 ± 0.1	15.1 ± 0.1	107.4
Roots	3.1 ± 0.1	1.98 ± 0.03*	64.3	11.2 ± 0.1	13.3 ± 0.2*	119.3
IK107VS$_3$/66						
Aboveground part	5.7 ± 0.2	2.9 ± 0.1*	50.1	24.5 ± 0.4	26.8 ± 0.3	109.5
Roots	4.1 ± 0.2	1.98 ± 0.01*	48.1	23.7 ± 0.1	24 ± 1	100.6

tolerance only during a short drought, because a long drought inhibits the enzyme regardless of genotype tolerance to this stress. That is why sucrose-phosphate synthase activity and sucrose content were studied in sprouts of corn lines with different level of drought tolerance under a short joint impact of water deficit and hyperthermia. The study showed that sucrose-phosphate synthase was positively activated under stress factors in drought-tolerant corn lines both in the aboveground part and roots of the plants (143 - 217% of reference value – Od329, 110 - 114% of reference value – IK107zM) (Table 3). Stress factors in non-drought-tolerant corn lines inhibited sucrose-phosphate synthase activity in the aboveground part and roots of the plants (76,6 - 48% of reference value). Stress factors also affected the level of sucrose content in corn sprouts. For instance, activation of sucrose-phosphate synthase in drought-tolerant corn lines was accompanied by significant increase in sucrose content both in the aboveground part and in roots of the sprouts (140 - 225% of reference value). Inhibition of sucrose-phosphate synthase activity amid water deficit and hyperthermia in non-drought-tolerant corn lines had little effect on sucrose content in sprouts, though there was a tendency towards growth thereof (107 - 119% of reference value) (Table 3). In other words, activation of sucrose-phosphate synthase and sucrose accumulation amid water deficit and hyperthermia in corn depend on the genotype's drought tolerance and may serve as auxiliary criteria for assessment of prospective drought tolerance of corn lines.

4. Concluding Remarks

Therefore, it has been established that corn lines with positively different levels of drought tolerance are characterized by varying activity of soluble lectins, content of abscisic acid, activity of sucrose-phosphate synthase and sucrose content in tissues of the sprouts under adverse abiotic factors (water deficit and hyperthermia). Activation of soluble lectins, sucrose-phosphate synthase and accumulation of abscisic acid in corn amid water deficit and hyperthermia depend on the line's drought tolerance and may be used as biochemical criteria for assessment of drought tolerance of corn lines.

Заключение

Итак, установлено, что линии кукурузы, которые достоверно отличаются по уровню засухоустойчивости, характеризуются неодинаковыми активностью растворимых лектинов, абсцизовой кислоты, сахарозофосфатсинтазы и содержанием сахарозы в тканях проростков при воздействии абиотических неблагоприятных факторов (водного дефицита и гипертермии). Активация растворимых лектинов, СФС и аккумуляция абсцизовой кислоты в условиях водного дефицита и гипертермии в растениях кукурузы зависят от уровня засухоустойчивости линии и могут быть использованы в качестве биохимических критериев при оценке уровня засухоустойчивости линий кукурузы.

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Creation of antifungal antibiotics of new generation by chemical modification and genetic engineering methods – Modern approach to a solution of the problem of an antibiotic resistance at candidiasis infections

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Abstract

This review is dedicated to the research of modified antifungal antimycotics against candidiasis infectious. One of the key and urgent problems in modern medicine and pharmacology in recent decades has become the problem of ineffective action of antibiotics or the development of antibiotic resistance. Immunity of microorganisms to the action of antibiotics has led to the search for new effective drugs. Treatment of fungal infections, especially candidiasis, is also one of the most important problems in modern medical mycology. The solution of this problem is in the creation of new forms of antifungal drugs that will be more effective than their predecessors. Molecular transformation of antimycotics can be carried out by the help of chemical modification and genetic engineering. Chemically modified antifungals of new generation have already been tested. Among them pimaricin, amphotericin B, nystatin and lucenzomycin. The positive effect of these drugs on test-cultures of different types of candidiasis in vitro was shown.

Аннотация

Настоящий обзор посвящен исследованию модифицированных противогрибковых антимикотиков против инфекционного кандидоза. Одной из ключевых и актуальных проблем современной медицины и фармакологии в последние десятилетия стала проблема неэффективного действия антибиотиков или антибиотикорезистентности. Иммунитет организмов к действию антибиотиков привел к поиску новых эффективных препаратов. Лечение грибковых инфекций, особенно кандидоза, также является одной из важнейших проблем современной медицинской микологии. Решением этой проблемы стало создание новых форм противогрибковых препаратов, которые будут более эффективны, чем их предшественники. Молекулярная трансформация антимикотиков осуществлялась с помощью химической модификации и генной инженерии. Доступность молекул антибиотиков для химической модификации функциональными аминными и карбоксильными группами и создание производных с использованием генной инженерии позволяют получать новые лекарственные препараты с улучшенными физико-химическими свойствами для более целесообразного использования в клинике. Были испытаны химически модифицированные противогрибковые препараты нового поколения. Среди них пимарицин, амфотерицин В, нистатин и люцензомицин. Показано положительное влияние этих препаратов на тест-культуры различных видов кандидоза in vitro. Суммируя представленные данные, хотелось бы отметить, что создание новых химически модифицированных и генно-инженерных препаратов с более эффективными терапевтическими параметрами открывает новые перспективы для решения проблемы антибиотикорезистентности.

Keywords: Macrocyclic compounds, polyene antibiotics (PA), candidiasis, antibiotic resistance

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Irrational use of antibacterial antibiotics with a wide spectrum of action leads to fungal infections, where the main indicator is the development of resistance to pathogens of invasive mycoses[1,2]. Since the treatment of fungal diseases, especially invasive mycoses, is mainly associated with polyene antibiotics, which are the products of microorganisms from *Streptomyces genus* (Actinomycetes), the solution to this problem was in the formation of new forms of antifungal, that should be more effective drugs. This problem is acute in the treatment of fungal infections that were previously treated with polyene antibiotics (PA), such as amphotericin, nystatin, trichomycin and candidin. Due to changes in the environmental situation in nature, there are new forms of organisms possessing genetic mutations[3]. Accordingly, the compounds produced by them and their properties change in response to the selective pressure.

One of these changes is the resistance of organisms to these drugs, that is, they become antibiotic resistant. Thus, treatment of invasive fungal infections is becoming problematic. The most common of the mycoses is currently candidiasis caused by various representatives of the genus *Candida*. The solution of this problem is based on the search of new pharmaceuticals that can be obtained by the methods of genetic engineering and chemical modification of these molecules. Some chemically modified antibiotics of new generation such as pimaricin, amphotericin B, nystatin and others were investigated. Testing of benzyl derivatives of pimaricin on six cultures of the *Candida* showed that the greatest antifungal activity took place if the chemical radical in the molecule is a nitrogroup or halogen in the phenyl ring. These derivatives are more efficient against causative agent of candidiasis than initial pimaricin. The research of nanoderivatives of nystatin and pimaricin concerning a number of test cultures of the *Candida* was very efficiently conducted. These nanoderivatives have high antifungal activity in relation to fungi and considerably increase stability and biopharmaceutical properties of these antibiotics in relation to causative agents of candidiasis on the basis of the tested 11 species of the *Candida* – *C. albicans, C. utilis, C. tropicalis, C. crusei, C. glabrata, C.lusitaniae, C.lipolytica, C.norvegensis, C.parapsilosis, C. kefyr* and *C. guillermondii*[4]. Thus chemical modification of PA makes it possible to obtain less toxic derivatives of antibiotics with improved chemotherapeutic properties and with expanded spectrum of biological activity[5]. The search of pharmaceuticals with improved therapeutic properties led to the new modifications of most efficient antimycotic – amphotericin B. Initial amphotericin B has widest range of application and is one of most researched and used in practical medicine antifungal macrolide antibiotics. Various derivatives of amphotericin B and nystatin in liposomal form – lipid complexes and colloidal dispersed forms – have also been developed[6]. New liposomal amphotericin derivatives with low toxicity and high resistance have been developed[7], Amphotericin B kills pathogens of fungal infections by binding to the ergosterol of fungi. Modifying the structure of amphotericin B, it is possible to obtain its derivative, which would have the ability to bind only to ergosterol, but not to cholesterol. Modified version of amphotericin B can be synthesized from a natural product into three stages: with a total yield of up to 25%.

Modification of amphotericin B by benzoxaborols was carried out either by carboxyl group at the carboxyl group macrolactone ring at position C16 or at the amino group of the amino sugar (Figure 1). A series of hybrid compounds – mono- and dimodified derivatives of amphotericin B – are synthesized. The study of biological activity of these compounds revealed in most of them the high antifungal activity in vitro against *Candida* yeast cultures. The greatest activity was shown by the dimodified borol derivatives for which modification on a carboxyl group of C16 dimethylaminoethylamid was used (Figure 1). On some results, in particular, on activity, they surpassed initial amphotericin B[8]. Because of nephro- and gematoxicity attempts were done to modify its low-toxic derivatives on the basis of methods of chemical synthesis and genetic engineering[8,9]. New genetically engineered polyene macrolides were obtained as a result of genetically engineering experiments with the strain of microorganism *Streptomyces noursei*. The research of the molecular and genetic mechanism of action showed that under the influence of a liposomal form of amphotericin B there is a depression of biofilms formation by fungi of *Candida albicans* along with the block of a gene of MET3 expression. After daily incubation of biofilms with liposomal amphotericin B there was no MET3 gene mRNA transcribed that indicates the block of this one. Results of experiments show that the use of liposomal antimycotics is highly efficient concerning fungi of *Candida albicans* and give the chance to predict their application for increase in efficiency of pharmacological effect of antifungal medicines and decrease of their therapeutic dose. It should be noted that currently there are many low-toxic highly efficient semi-synthetic derivatives of PA. The greatest role belongs to nanotechnology, because nanotechnology research in medicine is based on the creation of a new generation of drugs that differ in a more effective way of their delivery to organs and tissues[10,11]. The selection of nanoderivatives of macrolide antibiotics with efficient antifungal activity was very important for the treatment of mycoses. The action of

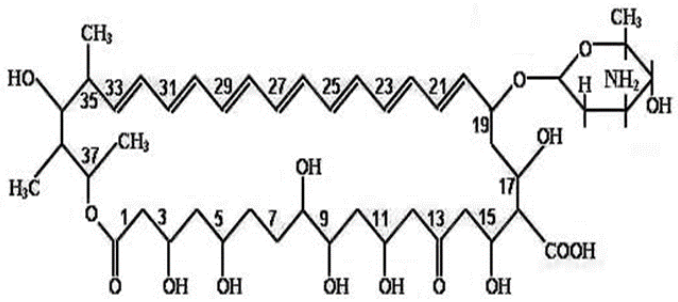

Figure 1 | The chemical structure of initial amphotericin B. Рис.1 Химическая структура исходного амфотерицина В.

nanoderivaives of tetraene PA nystatin and pimaricin was investigated on some test-cultures of yeast fungi *Candida*. It has shown that the above nanoderivatives have high antifungal activity to the pathogens of *Candida genus - C. albicans, C. tropicalis, C. crusei, C. glabrata, C. portugal, C. lipolytica, C. norvegensis, C. parapsilosis, C. akefyr and C. guillermondii*.

Thus, according to the results of different studies, it was shown that nanoderivatives of tetraene PA essentially rise stability and biopharmaceutical properties of these antifungal antibiotics[4,12].

Tetraene macrolide antifungal antibiotic lucenzomycin was first used by Italian researchers in the therapy of mycoses [13]. Lucenzomycin was obtained by microbiological synthesis from the organism Streptomyces lucensis. This antibiotic due to high toxicity has not been found an application in the drug therapy of mycoses, unlike nystatin and pimaricin. An obstacle in the systematic use of PA in medical practice is a relatively high toxicity (mainly nephrotoxicity), instability in storage, low solubility in water, as well as a decrease in sensitivity to pathogenic fungal microorganisms[14,15]. With this purpose we have synthesized low-toxic hydrophosphoryl sensimilla derivatives with high biological activity[15]. Studies on the search of new semisynthetic derivatives of lucenzomycin were continued and, as a result, relevant dialkylamidophosphate derivatives of this one have been obtained. Experiments on mice have shown that the acute toxicity of amidophosphate derivatives of lucenzomycin is 5 times less than that of the original antibiotic (LD50) and varies from 185 to 200mg/kg. Moreover, derivatives of lucenzolycin similar to nanoderivatives of pimaricin also have high antifungal activity against 11 test-cultures of yeast fungi of the Candida. It should be noted that amidophosphate derivatives contain in their molecules different chemical radicals $–CH_3$; C_2H_5; $CH_3(CH_2)_3$; C_6H_5; $\{Si(CH_3)_3\}_2$. It was shown that antifungal activity of first, forth and fifth compounds (i.e .with radicals CH_3, C_6H_5 and $\{Si(CH_3)_3\}_2$) exceeded activity of the original lucenzomycinin effects on *C. tropicalis, C. glabrata, C. parapsilosis* and *C. crusei*. Antifungal activity remained on the level of initial antibiotic against *C. albicans, C. utilis* and *C. guillermondii*. Activity of lucenzomycin derivatives is higher against these test-cultures[8,15-17]. However the action of amidophosphate derivatives of lucenzomycin on another *Candida* test-cultures (*C.lusitaniae, C.lipolytica, C.norvegensis, C. кefyr*) is less than that of original antibiotic [15-17]. For a long time, the causative agent of 90 % of *Candida* infections was considered *C. albicans*[16]. However in the recent years there has been an increase of Candida infections caused by *C. tropicali, C. parapsilosis, C. crusei, and C. glabrata*[1,17]. The high antifungal activity of some chemically modfied derivatives of lucenzomycin and their significantly low toxicity and better solubility in the water are important indicators that improve the biological, pharmacological and physical and chemical properties of these compounds in comparison with original antibiotic.

Concluding Remarks

A study of benzyl derivatives of pimaricin in six cultures of the genus *Candida* showed that these derivatives are more efficient against pathogens of candidiasis than initial pimaricin. Nanoderivatives of pimaricin and nystatin have high antifungal activity against these pathogens and significantly increase the stability and biopharmaceutical properties of these antibiotics in relation to the ones. The study of mono- and dimodified derivatives of amphotericin B show high antifungal activity in vitro against yeast cultures *Candida*. The use of liposomal antifungals is high efficient relative to the *Candida albicans* and make it possible to predict their use to improve the efficiency of the pharmacological action of these drugs and reduction of their therapeutic doses.

Thus the research of PA properties showed that biological activity is in the sharp dependence on chemical structure of molecules of these compounds[18,19]. The availability of antibiotic molecules to chemical modification by functional amine and carboxyl groups and the creation of derivatives using genetic engineering allow to obtain new pharmaceuticals with improved physical and chemical properties for more appropriate use in the clinic. Summing up the above data, we would like to note that the creation of new chemically modified and genetically engineered drugs with more effective therapeutic parameters opens up new prospects for solving the problem of antibiotic resistance.

Заключение

Исследование бензильных производных пимарицина в шести культурах рода Candida показало, что эти производные более эффективны против возбудителей кандидоза, чем исходный пимарицин. Нанопроизводные пимарицина и нистатина обладают высокой противогрибковой активностью по отношению к этим возбудителям и значительно повышают стабильность и биофармацевтические свойства этих антибиотиков по отношению к ним. Изучение моно-и димодифицированных производных амфотерицина В показало высокую противогрибковую активность in vitro в отношении дрожжевых культур Candida. Применение липосомальных противогрибковых препаратов является высокоэффективным по отношению к Candida albicans и позволяет прогнозировать их применение для повышения эффективности фармакологического действия этих препаратов и снижения их терапевтических доз. При этом исследование свойств ПА показало, что биологическая активность находится в резкой зависимости от химического строения молекул этих соединений.

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