MONITORING OF BIOLOGICAL PROPERTIES OF *PSEUDOMONAS AERUGINOSA* ISOLATES DURING LONG-TERM STORAGE

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Purpose of work is monitoring of biological properties of P. aeruginosa isolates in storage up to 3 years.

Bacteriological tests were performed according to generally accepted methods. For research we selected 5 P. aeruginosa cultures with typical biological properties isolated from poultry corpses. Isolates were stored on beveled agar under rubber stopper at + 4+5 °C up to 3 years. After the end of the research was examined morphological, cultural, biochemical, pathogenic properties. We studied the presence of pigments after prolonged storage and repeated passages on artificial media.

The color of culture has changed from green to brown at the long-term storage. After 3 years of storage all isolates were viable. However, pigments were absent in all cultures. After passage through biological organism production of pigments was resumed. But after 4-5 passages pigment is not formed. At Endo medium all cultures have a light pink colony. It is noted that after 5-6 passages isolates had growth at the same time in the form of S-, and R-colonies. All cultures have typical the biochemical properties: oxidized glucose and galactose; were inert to mannitol, sucrose and lactose. Catalase test was positive for all isolates.

P. aeruginosa isolates were viable for 3 years of storage on beveled agar under rubber stopper at + 4+5 °C. The formation of pigments during long-term storage and after reusable replanting on artificial media was suppressed. Pathogenic, basic biochemical and enzymatic properties did not change.

Keywords: *isolates of P. aeruginosa, long-term storage, pigments, pyocyanin, pioverdyn, fluorescence*

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CELLULAR PRION ISOFORMS LEVEL AND ATPASES ACTIVITIES IN THE CEREBELLUM OF DIFFERENT AGE WISTAR LINE RATS

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Prion infections are lethal diseases of the central nervous system in the humans and animals, the causative agent of which is the abnormal (infectious) prion (PrP^{Sc}, Sc – scrapie from). However cellular prion (PrP^{C}, C – cellular from) is a substrate for the PrP^{Sc} conversion. It is located on the outer surface of the cell membrane and involved in the maintenance of Ca^{2+}-homeostasis and other metabolic processes.

The age dynamics of PrP^{C} molecular isoforms quantity in the laboratory animals' cerebellum was determined. The increasing of three glycoforms level in the sixmonths animals' tissue and its decreasing in the thirtymonths animals' tissue was observed. These changes are closely correlated with the activity of transport enzymes, including Na^{+}/K^{+}- and Ca^{2+}-ATPases ($r = 0.825–0.857$).

Based on the results of the kinetic analysis we can concluded that ATP hydrolysis reaction by the studied enzymes in old animals the was less intense and longer, and the reaction product was accumulated in the smaller concentration than in the one- and six months animals.

Keywords: *cellular prion, western blotting, Na^{+}/K^{+}- and Ca^{2+}-ATPases, cerebellum, age changes*

Prions are infectious protein particles that cause the central nervous system fatal disorders of humans and animals – transmissible spongiform encephalopathies (TSE) [1, 2]. It is known the infectious (abnormal, PrP^{Sc}) and physiological (cellular, PrP^{C}) prions. Getting into the body, the pathological prion interacts with the cellular prion and as a result the conformation of PrP^{C} molecule is changing [3, 4]. The diseases occur not only as a result of infection but can be sporadically especially in older persons.

Based on the results of many studies of prion infections cellular models, the relationship between the prion disease and Ca^{2+}-exchange violation have been shown. There are two hypotheses concerning to the way of Ca^{2+}-homeostasis regulation by PrP^{C}. The first of one suggeststhat PrP^{C} directly interacts with the systems (Ca^{2+}-channels or metabotropic receptors) which provide the maintenance of Ca^{2+}-homeostasis, and modulate the activity of these systems. According to the second hypothesis, the PrP^{C} is part of the complex of multi-cell surface signaling that regulates the specific mechanisms and involved in control the expression of Ca^{2+}-transport proteins [5, 6]. However, there is no information about the PrP^{C} effect on ATPases activities, which are also involved in the calcium transport.

The aim of the study was to determine the correlation relationship between the ontogenetic changes of PrP^{C} level and activities of ATPases in the rats' cerebellum.

Materials and Methods. Manipulation with the animals were carried out under the principles of the „European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (Strasbourg, 1986), the Decision of the First National Congress on Bioethics (Kyiv, 2001) and the Law of Ukraine „On Protection Animals from Brutal Treatment" (Kyiv, 2006).

Research was carried out on the males of laboratory rats *Rattus norvegicus var. alba, Wistar line*, which were held under standard vivarium conditions. The animals aged one, six and thirty months were decapitated under ether anesthesia, the cerebellum was selected for this research.

A western blotting analysis of the cerebellum was carried out. For that, the tissue was homogenized and lysed in a special buffer as well as centrifuged for 2 min at 12.000 × g and at 4 ºC. The proteins were fractionated by electrophoresis in 12% gradient polyacrylamide gels (PAGE). The electro blotting of proteins on PVDF-membrane was carried (*Millipore*, USA). The samples with the same concentration of the protein were deposited in each PAGE well. The membranes were incubated with monoclonal primary antibodies (Antibody mAB6H4; *Prionics*, Switzerland) at +4°C for 12 h, and secondary polyclonal goat anti-mouse antibodies which are conjugated with alkaline phosphatase (*Sigma*, Germany) at +22°C during 60 min. Detection of the immune complexes was carried out using a substrate for alkaline phosphatase CDP-Star (*Tropix*, UK). Visualization was performed using X-ray film Retina XBM (*Lizoform Medical*, Ukraine) and film development kit for films (*Kodak*, Japan) [15].

To determine the Na^+/K^+- and Ca^{2+}-ATPases activities the tissues samples were homogenized for 1-2 min at Omni GLH-220 homogenizer in the sucrose medium. As a result of repeated centrifugation the tissue membrane fraction was obtained [16, 17], in which the studied parameters were determined. The activity of Na^+/K^+-ATPase was determined in the incubation medium. The activity of Na^+/K^+-ATPase was calculated by the difference between the total activity and ouabain insensitive activity, which was determined in medium with 1 mM of ouabain (selective inhibitor of Na^+/K^+-ATPase) (*Sigma*, Germany). The activity of plasma membrane Ca^{2+}-ATPase (PMCA) was calculated by the difference between activities that determined in the medium with 1 mM of thapsigargin (selective inhibitor of Ca^{2+}/Mg^{2+}-ATPase) (*Sigma*, Germany) in the presence and absence of Ca^{2+}. The activity of sarco (endo) plasmic reticulum Ca^{2+}-ATPase (SERCA) was calculated by the difference between the activities that were determined in medium with calcium ions in the absence and presence of thapsigargin. The measure of enzyme activity was inorganic phosphate (P_i) concentration [18].

The study of enzymatic reactions kinetic properties was carried out in a standard incubation medium, which was modified by the physical and chemical characteristics or certain components composition (incubation time, concentration of protein, ATP, Na^+, K^+, Ca^{2+}). The imaginary kinetic parameters that characterize P_i release reaction during ATP hydrolysis were determined [18].

The level of sodium and potassium ions in tissues was determined using the commercial kits (*Felicity diagnostics*, Ukraine) [20] and the level of total calcium was determined using atomic absorption spectrophotometer C-115M [15].

Student coefficient was calculated to assess the probable difference between the statistical characteristics of alternative data set. The accurate approximation was when P≤0.05 [12]. Statistical analysis of the results was carried out using the programs Excel and Origin.

Results. PrP^C has three glycoforms of: the diglycosylated (35–38 kDa), partially (mono) glycosylated (23–27 kDa) and nonglycosylated (19–21 kDa). The amount of these isoforms was determined by the result of western blot analysis of different age rats' cerebellum. In particular, the di-, mono- and nonglycosylated forms levels in the one-month rats' cerebellum were, respectively, 57, 55 and 7 standard units (Fig. 1*A*). The diglycosylated PrP^C form level was increased by 17% in the six months rats' tissue compared to one month age rats. Its level was decreased by 30% in old rats' cerebellum compared to mature animals. The nonglycosylated PrP^C form level was increased by 63% in the six months rats' tissue and was unchanged in the thirty months rats' tissue (Fig. 1*B*).

Similar results are described by Mar Cuadrado-Tejedor et al. [22]. The authors have analyzed the cortex and hippocampus areas of the rats' brain by the Western blotting analysis. The PrP^C level was increased in both areas in mature animals (38 weeks) compared to young (six weeks), and it is decreased in old animals (56 weeks). Moreover, the amount of nonglycosylated forms of the protein increased in the age of animals.

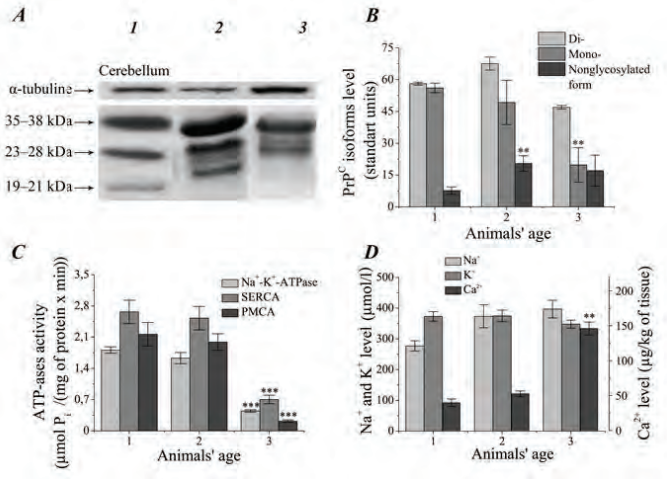

Figure 1. The analysis of the cerebellum of different age rats (*A* is the western blotting of PrP^C isoforms level; *B* is the histogram; *C* is the ATP-ases activities and *D* is the ions level): *1* is one month; *2* is six months; *3* is thirty months; (M ± m; * P < 0.05; ** P < 0.01; *** P < 0.001, the second age group of rats is compared to the first group and the third age group is compared to the second age group)

The activity of Na^+/K^+- and Ca^{2+}-ATPases and the ions (Na^+, K^+, Ca^{2+}) level in the different age rats' cerebellum was determined. The enzymes activity decreasing by ~80% in the thirty months animals' tissue was demonstrated. The sodium and potassium levels were unchanged, instead Ca^{2+} level was increased by 64% compared to the six months animals' cerebellum (Fig. 1*C*, *D*).

Kinetic characteristics of ATP hydrolysis in the cerebellum were also changing with animals' age increasing. The values of V_0 and P_{max} were decreased, respectively, by 67 and 77% but the value of τ was increased by 15–49%. The V_{max} value under these conditions was lower in 2–16 times, while K_m value was lower by 1.2–82% which indicates a growing affinity to the enzyme substrate (ATP) (Table 1). In the thirty months animals' cerebellum the Ca^{2+}-ATPases remain its activities under high calcium ions concentration in the medium. It should be noted that the ions concentration value optimum for the Na^+/K^+-ATPase is shifting towards in the increase of Na^+ level which is consistent with a slight increase of these ions in the tissues as a whole.

Table 1 – Kinetic parameters of ATP hydrolysis in the cerebellum

Kinetic parameters	Enzymes	Animals' age, months		
		1	6	30
V_0 (P_i μmol / (mg of protein×min))	Na^+/K^+-ATPase	0.826	0.722	0.136***
	SERCA	1.209	0.709*	0.325**
	PMCA	1.088	0.861	0.159***
P_{max} (P_i μmol / mg of protein)	Na^+/K^+-ATPase	1.551	0.883*	0.321**
	SERCA	3.183	3.461	0.810***
	PMCA	2.590	2.453	0.215***
τ (min)	Na^+/K^+-ATPase	1.878	1.223*	2.356*
	SERCA	2.632	2.883	3.492
	PMCA	2.381	2.848	3.352
V_{max} (P_i μmol / (mg of protein×min))	Na^+/K^+-ATPase	0.532	0.817*	0.425*
	SERCA	9.083	14.124*	1.130***
	PMCA	4.564	4.032	0.256***
K_m (mmol/l)	Na^+/K^+-ATPase	0.526	0.985*	0.997
	SERCA	6.837	12.476*	2.235***
	PMCA	3.083	2.804	0.652***

Comment: V_0 is initial reaction velocity; P_{max} is maximum amount of reaction product; τ is reaction time; V_{max} is maximum velocity of enzymatic reaction; K_m is Michaelis constant

By the results of correlation analysis a direct strong correlation between the PrP^C level and ATPases activities was carried out (r=0.825–0.857) as well as between the activities of these enzymes with each other (r=0.998–0.999) and between the ATPases activities and ions level (r=0.739–0.999) but it was inverse for Na^+ and Ca^{2+}.

Thus, a correlation between the cellular prion level and ion transporters activity, including the Na^+/K^+- and Ca^{2+}-ATPases, in the different age rats' cerebellum was shown in the first time. Perhaps, this dependence is due to similar functions and localization of studied proteins in the body.

Conclusions. 1. The PrP^C molecular isoforms level is increasing with animals' age. PrP^C level is the smallest in young animals' cerebellum, it significantly increases in six months animals and it decreases in thirty months animals.

2. The activities of Na^+/K^+- and Ca^{2+}-ATPases in cerebellum decrease depending on the animals' age increasing. When the calcium is accumulated the sodium and potassium levels are not significantly changed in old rats' tissue. As a result of kinetic analysis, a less intensity and longer lasting of hydrolysis reaction in older animals compared to one and six months animals were shown.

3. There is a correlation between the ontogenetic changes of PrP^C level and transport enzymes activity in the different age animals' cerebellum.

Prospects for future research is to identify the relationship between the cellular prion level and ATPases activity in other organs of rats' prion replication system.

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ВМІСТ ІЗОФОРМ КЛІТИННОГО ПРІОНА Й АКТИВНОСТІ АТФ-АЗ У МОЗОЧКУ ЩУРІВ ЛІНІЇ WISTAR РІЗНОГО ВІКУ

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Мета роботи визначення кореляційного взаємозв'язку між онтогенетичними змінами вмісту клітинного пріона (PrP^C) та активністю АТФ-аз у мозочку щурів.

Методи. Дослідження проводили на самцях лабораторних щурів Rattus norvegicus var. alba, лінії Wistar. Тварин, віком один, шість та тридцять місяців, декапітували під ефірним наркозом і відбирали мозочок для досліджень. Проводили вестерн блот аналіз цієї тканини. Визначали активності Na^+–K^+ та Ca^{2+}-АТФ-аз. Дослідження кінетичних властивостей ензиматичної реакції проводили у стандартному середовищі інкубації, яке модифікували за фізико-хімічними характеристиками або складом певних компонентів.

Результати. Вміст глікоформ PrP^C: диглікозильованої (35–38 кДа), частково (моно-) глікозильованої (23–27 кДа) та деглікозильованої (19–21 кДа), збільшувався у тканині шестимісячних щурів, порівняно з одномісячними. У мозочку старих тварин їх вміст знижувався, порівняно зі зрілими.

Встановлено зменшення активностей ензимів у тканині тридцяти місячних тварин на ~80 %, вміст іонів натрію і калію достовірно не змінювався, тоді як Кальцію – зростав на 64 %, порівняно зі шестимісячними тваринами. За цих умов значення V_0, P_{max}, V_{max} та K_m зменшувалися, проте т збільшилося.

Між вмістом PrP^C та активністю ензимів АТФ-аз встановлена пряма сильна кореляція (r=0,825–0,857).

Висновки. Вміст PrP^C є найменшим у мозочку молодих тварин, зростає шестимісячних та зменшується – у тридцяти місячних. Активність АТФ-аз знижується зі зростанням віку тварин. Існує кореляційна залежність між онтогенетичними змінами вмісту досліджуваного протеїну та активності ензимів-транспортерів у мозочку лабораторних тварин.

Ключові слова: *клітинний пріон, вестерн блот, Na^+–K^+- та Ca^{2+}-АТФ-ази, мозочок, вікові зміни*