Кальве — Пертеса. Проблема актуальна ввиду распространенности заболевания. На основании анализа и синтеза текущей и ретроспективной научной литературы авторами рассмотрены и классифицированы основные методы лечения по принципам действия, практическому применению, а также дана оценка эффективности с точки зрения доказательной медицины.

რეზიუმე

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ლიტერატურის მიმოხილვაში გაანალიზებულია თანამედროვე და რეტროსპექტული სამეცნიერო ლიტერატურა ლეგა-კალვე-პერტესის დაავადების სამკურნალო მეთოდების შესახებ. განხილულია მკურნალობის ოპერაციული და კონსერვატიული მეთოდები, რომლებიც კლასიფიცირებულია მკურნალობის, მოქმედების პრინციპების და პრაქტიკული გამოყენების გათვალისწინებით. განხილული მასალის ანალიზის და სინთეზის შედეგად ავტორებს მოცემული აქვთ მეთოდების ეფექტურობის შეფასება.

STRESS-AFFECTED Akt/mTOR PATHWAY UPREGULATED BY LONG-TERM CREATINE INTRAPERITONEAL ADMINISTRATION

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Oxidative stress is known to be characterized by significant alterations in metabolic processes, namely changes in the hormonal status, decreased energy metabolism and antioxidant status, as well as quantitative changes in enzyme activity and signalling molecules, which, in turn, affect transcription and translation processes [20,24]. Several compounds can prevent these processes. Among them is the Creatine (Cr; α-N-methylguanidinoacetic acid), which can be found in almost all mammals. It is primarily concentrated in muscle and brain. It participates in the Cr/CK/PCr system, is actively involved in energy metabolism, and its deficiency is associated with a decline in many physical and cognitive functions [2,9,13]. It is believed that the primary mechanism of action of Creatine (Cr) is its participation in the energy storage processes. Besides, various experiments also confirmed its neuromodulatory and neuroprotective properties [4,17]. Cr synthesized in nerve cells functions as a signalling molecule. In particular, it can activate some signalling pathways and, in this way, regulate energy metabolism, influencing growth, proliferation and viability of the cell [1,14]. In the brain, Creatine is most concentrated in the regions associated with learning processes and memory (such as Hippocampus, Pyramidal neurons of the cortex, Purkinje cells of the cerebellum). It is assumed that these areas are also marked with high ATP metabolism [21].

Cr is not only established to be synthesized by neurons, but it is also suggested to be delivered peripherally through the blood-brain barrier [2,4]. Exogenous Cr showed its neuroprotective properties in the number of neurological diseases such as Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS), head injuries [3]. Quantitative changes in Cr have also been shown in various psychiatric disorders, such as depression [1,5].

Recent data have further revealed the antioxidant properties of Cr [14,29]. Observations have shown that lipid peroxidation processes are down-regulated as antioxidant enzymes are activated in muscle and central nervous system (CNS), during Cr supplementation [14]. Such alterations might be caused by several stressors, such as long-term violation of natural circadian rhythm [26]. This kind of stress is usually accompanied by a change in antioxidant and energy metabolism - resulting in ATP deficiency, the brain's energy potential and functional deterioration, cell viability reduction, stimulation of pro-apoptotic processes, and variation in ion content [32].

Considering the above mentioned, the purpose of our investigation was to study complications in energy metabolism in the hippocampus under stress caused by long-term disturbance of circadian rhythm and the preventive action of Cr administered exogenously.

Material and method. Experiments were conducted on 200–250 gr male Wistar rats. The animals were divided into three main groups before the experiment:

(1) G1 – control group – was kept in a common cage under natural conditions (dark/light ratio =10/14);

(2) G2 – stressed group – individuals were maintained in individual cages in the darkness (dark/light ratio = 23.5/0.5) for 30 days;

(3) G3 – Cr-treated stressed - individuals were maintained in individual cages in the dark (dark/light ratio = 23.5/0.5) for 30

days and were injected Cr during this period (see section: Cr Supplementation).

During the experiments, all the rats were given water and a standard laboratory chow *ad libitum*. The experiment was repeated for four independent series.

The experiments were conducted in full accordance with the legal and statutory acts applicable in Georgia and the international agreements ratified by the country, such as the Law of Georgia on Health Care and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Cr supplementation. Cr was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted in 5% dimethyl sulfoxide (DMSO). During 30 days, G3 animals were intraperitoneally (i.p.) injected 140 mg/kg/day. The rest of the experimental animals were supplemented with 5% dimethyl sulfoxide, depending on the animal weight (1 ml/100 gr). The Cr dose to be injected was chosen based on the data supplied by various authors based on their research [14,29].

Assessment of the CK activity. Creatine Kinase catalyzes the incorporation of phosphate into Creatine to form Creatine phosphate. The amount of free phosphate existing due to ATP hydrolysis in the mitochondria was evaluated in the Phosphovanadium-molybdate complex and analyzed by spectrophotometer. The reaction medium contained 100 µl of the suspension sample and 0.5 ml solution of Creatine (1.9 mM) prepared in special buffer (2.5mM glycine + 2mM Na_2CO_3 + 0.2 mM $MgSO_4$, pH 9.7). The resulting mixture was suspended for 5 min at 37°C; then 0.5 ml of ATP (0.07 mM) was added and further incubated at 37°C for 60 min. The reaction was stopped with the addition of a 14% solution of Trichloroacetic acid. The resulted solution was then centrifuged for 10 min at 3000 g. Finally, 0.5 ml of supernatant was mixed with 0.5 ml of an Ammonium Vanadate and Ammonium Molybdate mixture (1:1). The amount of phosphate was assessed by spectrophotometry at λ=400 nm [32].

Electrophoresis of proteins in polyacrylamide gel. The protein fractions were analyzed by SDS-PAGE. The same volume of buffer for electrophoresis (20% glycerol, 10% 2-mercaptoethanol, 6% SDS, 0,02-0,04% bromophenol blue 250 mM Tris-HCl pH 6.7) was added to each sample and boiled for 7 minutes. Electrophoresis was applied to 7.5-12% of the acrylamide/bisacrylamide gel until the complete separation of proteins.

Immunoblotting. For immunoblotting experiments 50 µg of protein was denatured at 90°C for 5min, separated by SDS-PAGE on 15% gels and transferred to nitrocellulose membranes. After blocking with 5% bovine serum albumin (BSA) and 0.05% Tween 20 in Tris–HCl buffered saline; the membranes were incubated with primary antibodies in the blocking solution. Immunolabeled bands were visualized using enhanced chemiluminescence (Amersham Biosciences) and analyzed by densitometric scanning. The intensities of the bands were within the linear range of the amount of protein loaded. The concentration of protein in the study samples was applied by Lowry protein assay.

All statistical analyses were conducted using SPSS software (version 23, SPSS, Chicago, IL). One-way ANOVA was used to assess group differences in all physiological and biochemical values. Tukey HSD or Games-Howell *post hoc* test was performed to assess the differences between groups. The values are expressed as mean±SEM. P values less than 0.05 were considered as statistically significant.

Results and discussion. *Changes in the activities of CK in the hippocampus cells under stress conditions caused by disruption of the circadian rhythm*

Fig. 1 shows that both CK of the hippocampus is quite sensitive to chronic stress. Especially mtCK, whose activity is reduced by about ≈ 50% under chronic stress (G2), compared to the control group (G1). However, it was also found that exogenous Creatine injections, increased their activity.

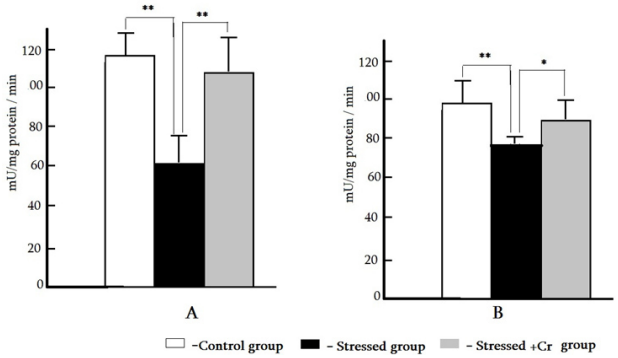

Fig. 1. Alterations of the CK activity in mitochondrial and cytoplasm fractions of the hippocampus in conditions of chronic stress. The results are expressed as mean ± SD.

notes: Control (G1), Stressed (G2), and Cr-treated stressed (G3) animals. The data are mean ± SEM of three individual series

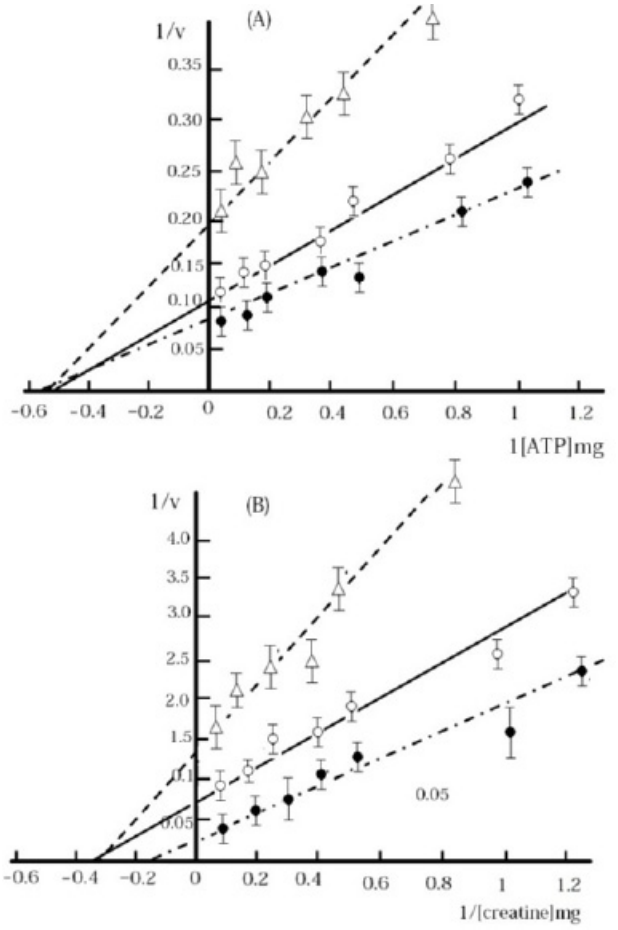

Fig. 2. Influence of exogenous Cr on the kinetic parameters (V_{max}, K_m) of Creatine Kinase among

○ – Control (G1); □ – Stressed (G2); ● – Cr-treated stressed (G3) animals.

note: x-axis – 1/V, y-axis – substrate concentration (mg/ml)

The results indicate that under the long-term violation of natural circadian rhythm, the rate CK decreased.

Changes in kinetic parameters of Creatine Kinase (CK) during the prolonged disruption of circadian rhythm. In further experiments, it was interesting to find out the reason that caused the energy metabolism enzyme activities changed under disruption of circadian rhythm and the basis of Cr action on these processes. This issue was investigated on the example of changes in kinetic parameters (V_{max}, K_m) of CK. The data obtained are shown in Fig. 2.

The obtained data showed that the enzyme's Vmax was reduced during the experimental conditions, and K_m was increased. These data made us think that the leading cause of the CK activity changes was reducing its amount, which is likely to be caused by a decrease in the synthetic reactions' intensity. However, Cr's administration increased V_{max}, that could be due to the enzyme's quantitative rise.

Impact of exogenous Creatine on PI3K / Akt / mTOR signalling pathway. The purpose of the further experiment was to study the intracellular signalling pathways that determine the hippocampus's energy metabolism under the prolonged disruption of circadian rhythm and detect Cr's preventive effects on its progression. In this regard, an essential part of the PI3K/Akt/mTOR signalling pathway, that represents one of the major regulators for energy metabolism and anabolic processes, were analyzed.

In the beginning, it was analyzed the qualitative changes of protein mTOR and its active, phosphorylated form in the hippocampus of G2 and G3 animals. It was observed that the amount of mTOR is significantly decreased in the hippocampal cells of G2 animals compared with that of the control group (G1). In contrast, in G3 individuals, this indicator's reliable increment was observed (Fig. 3A). Similar changes were also observed in the case of phosphorylated mTOR (Fig. 3B).

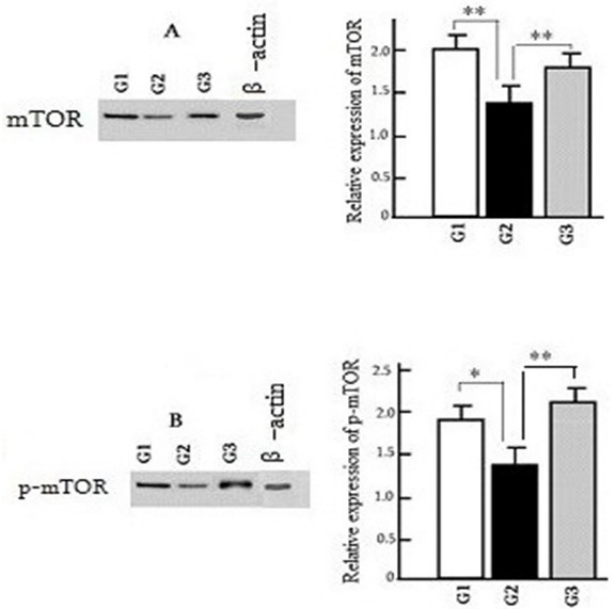

Fig. 3. Quantitative changes of mTOR (A) and phosphorylated mTOR (B) in the hippocampus cells under long-term disruption of natural circadian rhythm

notes: Control (G1), Stressed (G2), and Cr-treated stressed (G3) animals. Data are presented as means ± SEM (N=5)

In parallel with mTOR, another component of this signalling pathway was analyzed: the enzyme Akt (Protein kinase B; PKB). As Figure 4A and 4B shows, compared with the control group, there is no reduction for the enzyme in the hippocampus cells of the G2 group, although the quantity of phosphorylated Akt is low. On the other hand, exogenous supply of Cr increases the number of phosphorylated Akt, similar to mTOR during the disruption of the circadian rhythm.

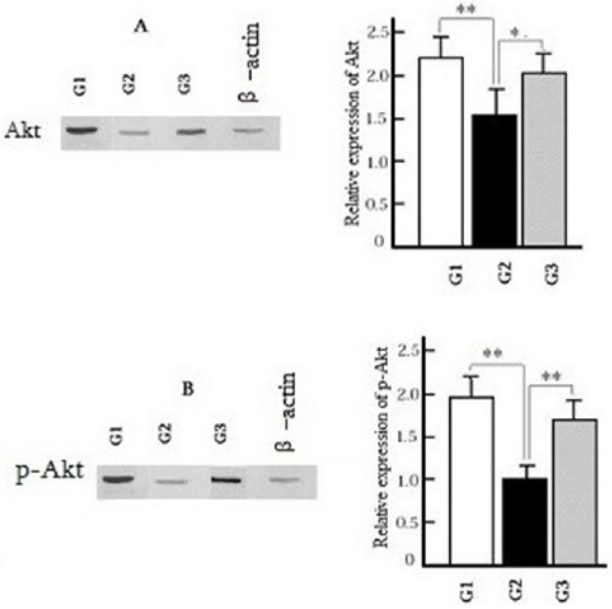

Fig. 4. Quantitative changes of Akt (A) and phosphorylated Akt (B) in the hippocampus under long-term disruption of natural circadian rhythm and case of i.p. supplementation of Creatine

notes: Control (G1), Stressed (G2), and Cr-treated stressed (G3) animals. Data are presented as means ± SEM (N=5)

Our previous experiments observed that daily injection of 140 mg/kg Creatine into rats for 30 days upregulated antioxidant enzymes activity. It is assumed that exogenous Cr promoted synthesis of the enzymes. It was also shown that Cr supplementation improved ATP level in the cells [18]. So, the research goal was to determine the mechanism by which Cr supplementation positively affects the hippocampus's energy metabolism under the long-term disturbance of the circadian rhythm.

It is well known that as a result of prolonged stress-induced oxidative processes, heightened ROS levels primarily affect mitochondrial enzymes due to their specific structural features. The variation in the enzyme activity is caused by their structural and quantitative changes [7]. Considering this, investigating the nature of differences in enzyme activity during the Cr's intraperitoneal administration was valuable. It was studied using the example of alterations in kinetic parameters (V_{max}, K_m) of Creatine Kinase. Figure 2 shows that oxidative stress reduces the V_{max} of the reaction and changes K_m, which indicates a quantitative reduction in enzyme and structural changes. It seems that the exogenous administration of Cr increases the activity of the enzyme at the expense of V_{max}, the reason likely being an increase in the amount of Creatine Kinase to be influenced by Creatine [12]. Similar results were seen in other studies. It is hypothesized that the Cr effect is caused by heightening energy efficiency of the cell and intensity of anabolic processes, expressed by a higher number of specific proteins, including enzymes [24]. However, there is also a different opinion, which says that the reason for this change is that Cr is tending to bind and neutral-

ize reactive radicals, and on the other hand, its direct action on certain enzymes [18]. The data indicate that increased intensity of synthetic reactions should cause an increase Creatine Kinase activity during exogenous administration of Cr.

The idea is strengthened by the results from observation on PI3K/Akt/mTOR pathway under the long-term disturbances of natural circadian rhythm and the impact of exogenous Cr supplementation. This process represents one of the major cellular signalling pathways regulating metabolism, apoptosis, and proliferation. Our attention was drawn to the target protein of rifampicin, mTOR, which has serine-threonine kinase activity. This protein is considered to be the primary regulator of energy metabolism and synthetic reactions [28]. mTOR is found in two protein complexes - mTORC1 and mTORC2 [15]. mTORC1 is mainly associated with lysosomes and is the primary regulator of protein synthesis, while mTORC2 is the activator of Akt, i.e. protein kinase B. Our data showed a reduction in total and activated mTOR in the hippocampus under stress conditions (Fig. 3). It should be noted that mTOR activity is essential for the mitochondrial respiratory chain [16].

mTOR activity in the cell is known to be regulated by both negative and positive signals. The negative regulator of mTOR is complex TSC1/2 (tuberous sclerosis complex ½) activated by various factors, including an increased number of ROS in the cell [31]. We established augmentation of the hippocampus's oxidation process under disrupted circadian rhythm and, therefore, a quantitative increase in active radicals [25]. Those mentioned above may be related to the reduction of the active mTOR concentration.

Besides the impact on mTOR activity, energy resources are also influenced by oxidative processes, such as the ATP level in the cell [33] that decreases under stress [10].

Data suggests that mTOR is activated by Akt (protein kinase B), which is an enzyme with serine-threonine kinase activity (Protein kinase B), which, in its turn, is activated by Phosphoinositole-3-Kinase (PI3K), followed by a change of PI3K/Akt/mTOR pathway. Remarkably, this signalling pathway's activity is changed by different extracellular signals, including stress factors [11,22]. Our data confirm that, in prolonged stress, when oxidative phosphorylation and energy metabolism decrease, the amount of phosphorylated Akt decreases (Fig. 4A, B).

Our findings show that the intraperitoneal administration of Cr into experimental animals improves the reduced energy potential and has a neuroprotective effect. The presented data show that with exogenous administration of Creatine (G3), the number of total and activated Akt molecules increases compared to those in G2 individuals (Fig. 4A, B).

Thus, the experiments' data show that the prolonged disruption of the natural circadian rhythm causes an impediment in the PI3K/Akt/mTOR signalling pathway. It is reflected in the process of protein synthesis and the quantitative reduction of creatine kinase. Consequently, it can be assumed that Creatine performs its positive role in hippocampal cells' energy metabolism via its modulatory effects on the PI3K/Akt/mTOR signalling pathway. This opinion certainly requires additional studies to strengthen the assumption of Cr's modulating effect in the central nervous system's functioning.

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SUMMARY

STRESS-AFFECTED Akt/mTOR PATHWAY UPREGULATED BY LONG-TERM CREATINE INTRAPERITONEAL ADMINISTRATION

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Disruption of natural circadian rhythm leads to the development of chronic stress. It provokes cellular metabolism changes, including a reduction in energy production and downregulation of anabolic reaction. Considering the importance of those processes, it is crucial discovering the substances that can prevent those stress-induced alterations. Our attention was drawn to Creatine.

The experiments showed that Creatine's intraperitoneal injections during a prolonged disruption of circadian rhythm help activate mitochondrial creatine kinase. Since the central regulatory substance in energy metabolism is the signalling molecule mTOR, we studied its quantitative changes under long-term disruption of circadian rhythm and exogenous creatine administration. The results revealed that Creatine's exogenous supplementation increases phosphorylated mTOR and its activator – Akt.

Consequently, it can be assumed that Creatine performs its positive role in hippocampal cells' energy metabolism via its modulatory effects on the PI3K/Akt/mTOR signalling pathway.

Keywords: circadian rhythm, oxidative stress, creatine, creatine kinase, PI3K/Akt/mTOR signalling pathway.

РЕЗЮМЕ

ВЛИЯНИЕ ЭКЗОГЕННОГО КРЕАТИНА НА AKT/MTOR СИГНАЛЬНЫЙ ПУТЬ В УСЛОВИЯХ ДЛИТЕЛЬНОГО СТРЕССА

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Нарушение естественного циркадного ритма приводит к изменениям клеточного метаболизма и развитию хронического стресса, что подразумевает снижение энергетического статуса клеток, а также интенсивности анаболических реакций.

Учитывая вышесказанное, крайне важно обнаружить вещества, которые могут предотвратить эти процессы во время хронического стресса. Эксперименты показали, что внутрибрюшинные инъекции креатина во время длительного нарушения циркадного ритма способствуют активации митохондриальной креатинкиназы в гиппокампе. Поскольку центральным регуляторным веществом в энергетическом метаболизме является сигнальная молекула mTOR, нами изучены ее количественные изменения при длительном нарушении циркадного ритма и влиянии экзогенного креатина на этот процесс.

Результаты показали, что введение добавки креатина увеличивает количество фосфорилированного mTOR, а также его активатора - Akt в организме. Авторы предполагают, что креатин выполняет положительную роль, благодаря своим модулирующим воздействиям на PI3K/Akt/mTOR сигнальнй путь.

რეზიუმე

ეგზოგენური კრეატინის ეფექტი ხანგრძლივი სტრესის პირობებში შეცვლილ Akt/mTOR სასიგნალო გზაზე

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ივ. ჯავახიშვილის სახ. თბილისის სახელმწიფო უნივერსიტეტი, ზუსტ და საბუნებისმეტყველო მეცნიერებათა ფაკულტეტი, ბიოლოგიის დეპარტამენტი, საქართველო

ცნობილია, რომ ბუნებრივი ცირკადული რიტმის დარღვევას თან სდევს უჯრედული მეტაბოლიზმის ცვლილება და ქრონიკული სტრესის განვითარება, რაც გულისხმობს როგორც უჯრედის ენერგეტიკული სტატუსის დაქვეითებას,ასევე ანაბოლური რეაქციების შემცირებას. ზემოაღნიშნულის გათვალისწინებით, მნიშვნელოვანია ისეთი ნაერთების მოძიება, რომლებსაც შესწევთ უნარი ქრონიკული სტრესის პირობებში მოახდინონ ამ პროცესების პრევენცია.

ექსპერიმენტში ნაჩვენებია, რომ ცირკადული რიტმის ხანგრძლივი დარღვევის პირობებში კრეატინის ინტრაპერიტონიალური შეყვანა ჰიპოკამპის უჯრედებში ააქტივებს სტრესის შედეგად დაქვეითებული მიტოქონდრიალური კრეატინკინაზას აქტივობას. იმის გათვალისწინებით, რომ უჯრედის ენერგეტიკული მეტაბოლიზმის მიმდინარეობის ერთ-ერთ ცენტრალურ რეგულატორად ითვლება mTOR, შესწავლილია მისი რაოდენობრივი ცვლილებები ხანგრძლივი ცირკადული რიტმის დარღვევის პირობებში და ეგზოგენური კრეატინის გავლენა ამ პროცესზე. მიღებულმა შედეგებმა აჩვენა, რომ ორგანიზმში კრეატინის შეყვანა ზრდის როგორც გააქტივებული mTOR-ის, ასევე მისი აქტივატორის Akt რაოდენობას.

აღნიშნულის გათვალისწინებით, შესაძლებელია ვივარაუდოთ, რომ კრეატინის დადებითი ეფექტი ხანგრძლივი ცირკადული რიტმის დარღვევის პირობებში განვითარებული სტრესის დროს ჰიპოკამპის უჯრედების ენერგეტიკულ მეტაბოლიზმზე გამოწვეულია მისი მოდულატორული მოქმედებით PI3K/Akt/mTOR სასიგნალო გზაზე.

FEATURES OF GRANULATION TISSUE MORPHOLOGY AROUND THE NET ALLOTRANSPLANT WHEN APPLYING POSTOPERATIVE RADIATION THERAPY

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Patients with oncological diseases of the abdominal organs are known to constitute the highest risk group for the postoperative eventration [1]. In order to prevent the development of the postoperative eventration, the majority of surgeons strengthens the anterior abdominal wall with mesh allografts, but the rate of regeneration and the risk of purulent-septic complications' development from the side of the postoperative wound in patients with cancer has certain features stipulated by the presence of tumorous intoxication, phenomenon of the secondary immunodeficiency cachexia, anemia, etc. [2-4]. The use of complex treatment, which includes postoperative radiation therapy, significantly slows down reparative processes in the irradiation area, that also increases the risk of eventration.

The study of the postoperative teleirradiation therapy influences on the morphology of granulation tissue around reticular allograft will allow to determine more optimally the expediency and safety of this type of treatment in strengthening the anterior abdominal wall in patients with abdominal cancer.

The objective of the article to study the peculiarities of the granulation tissue morphology around the elements of the reticular allograft of the muscular-aponeurotic layer of the anterior abdominal wall when using postoperative distant gamma therapy in the experiment.

Material and methods. The experiment was performed on 168 mature nonlinear rats of middle age of both sexes, weighing not less than 180 g, which were implanted with prolene (Prolene) reticular allograft of ETHICON company into the tissues of the muscular-aponeurotic layer of the anterior abdominal wall, according to the method proposed by us (Pat.106161 dated 25.04.2016) [5].

All experimental animals were divided into two groups – the group of comparison (72 rats) and the main one (96 rats). Animals of the main group, from the 13th to the 19th day after implantation of a reticular allograft, received distant gamma therapy on the organs of the abdominal cavity with gamma-therapeutic device AGAT - P1U isotope Co60, 1.25 MeV, by a single irradiating dose of 2 g, total irradiation dose - 14 g.

Taking of biological material was carried out on the 20th, 30th, 40th and 50th day after surgery, by excision of the muscular-aponeurotic layer of the anterior abdominal wall together with a reticular allograft, under general intravenous anesthesia (solution chloral hydrate 200-250 mg/kg).

The surgical procedures were performed in the vivarium of the Higher State Educational Establishment of Ukraine "Bukovinian State Medical University", in accordance with the national requirements of the "General Ethical Principles of Experiments on Animals" (Ukraine, 2011), which are in line with the Council of Europe Convention about protection of the vertebrate animals used for research and other scientific purposes (dated 18.03.1986).

For light optical examination, at histological investigation bioptates of the muscular-skeletal aponeurotic layer of the ante-