NATIONAL ACADEMY OF SCIENCES OF UKRAINE
Palladin Institute of Biochemistry

BIOTECHNOLOGIA ACTA

Vol. 15, No 2, 2022

BIMONTHLY

Editorial address:
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According to the resolution of the Presidium of the National Academy of Sciences of Ukraine from 27.05.2009 №1-05 / 2 as amended on 25.04.2013 number 463 Biotechnologia Acta has been included in High Attestation Certification Commission list of Ukraine for publishing dissertations on specialties "Biology" and "Technology".

Certificate of registration of print media КВ series №19650-9450ПР on 01.30.2013

Literary editor — H. **Shevchenko**; Computer-aided makeup — **O. Melezhyk**
Authorized for printing 29.04.2022, Format — 210×297. Paper 115 g/m^2. Gaqrn. SchoolBookC. Print — digital. Sheets 11.6. An edition of 100 copies. Order 6.6. Make-up page is done in Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine. Print — **O. Moskalenko** FOP

NATIONAL ACADEMY OF SCIENCES OF UKRAINE
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BIOTECHNOLOGIA ACTA

Scientific journal

Bimonthly

Vol. 15, No 2, 2022

REVIEWS

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НАЦІОНАЛЬНА АКАДЕМІЯ НАУК УКРАЇНИ
Інститут біохімії ім. О. В. Палладіна

BIOTECHNOLOGIA ACTA

Науковий журнал

Том 15, № 2, 2022

ОГЛЯДИ

ЕКСПЕРИМЕНТАЛЬНІ СТАТТІ

Тези конференції
«АКТУАЛЬНІ ПРОБЛЕМИ БІОХІМІЇ ТА БІОТЕХНОЛОГІЇ – 2022»

UDC 615.468 https://doi.org/10.15407/biotech15.02.007

CHARACTERISTICS OF WOUND INFECTIONS AND METHODS OF THEIR TREATMENT USING PREPARATIONS OF BIOLOGICAL ORIGIN

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Received 04.02.2022
Revised 09.04.2022
Accepted 29.04.2022

Surgical wound infections are the most common patients' complications in the postoperative period. In the modern clinic, they worsen the disease prognosis and remain the most important and acute health problem in all countries of the world.

The *aim* of the work was to analyze current scientific data on the peculiarities of the pathogenesis of wound infections and types of their pathogens, as well as drugs of biological origin in the treatment of wound infections.

The paper discusses in detail the problem of infection of wound injuries during surgery and domestic injuries of various kinds. The main pathogens of wound infections are considered. Specific pathogenicity factors for bacteria of the genera *Staphylococcus*, *Pseudomonas*, *Enterobacteriaceae* were analyzed. Based on the analysis of literature sources, a list of drugs of biotechnological origin that can be effectively used in combination therapy for the treatment and prevention of wound infections was determined.

Conclusions. The result is the identification of those mechanisms of pathogenesis of wound infections that determine the effectiveness of the use of drugs of biological origin in this pathology treatment.

Key words: wound infections, contaminations by microorganisms, *Staphylococcus, Pseudomonas, Enterobacteriaceae,* antibiotics, immunomodulatory.

The doctrine of the wound and the wound process is one of the current problems. The tendency to increase the number of patients in surgery and as a consequence, numerous surgical interventions increasing the complexity and duration of operations, as well as progressive antibiotic resistance of pathogenic microflora complicate the problem of prevention and treatment of wounds.

The increase in the number of purulent diseases and complications after surgery, the growth in cases of infection generalization and the existence of various toxic-allergic reactions, cause negative results in the treatment of patients with this type of pathology, which indicates the urgency of purulent infection in surgery.

According to the World Health Organization (WHO), 1.7% to 44.8% of patients in the European region have been diagnosed with nosocomial infections (NIS) in the last 25 years. Home and foreign studies have found that this type of infection is the most common form of complications of surgical treatment of acute surgical disease in the postoperative period. About 17–35% of the operated patients' cases was diagnosed. The frequency of surgical wound infections directly depends on the type and nature of surgical intervention: for clean wounds it is 1.5–6.9%, conditionally clean – 7.8–11.7%, contaminated – 12.9–17%, when the same for "dirty" it is 10–40%.

Although the type of infection in patients in different countries around the world may be

influenced by different factors, it is generally accepted that every tenth patient who seeks medical attention is affected by NIS.

It was also found that in surgical hospitals in Ukraine an average of 13.4–38.8% of STIs are found among patients during the postoperative period, which correlates with those of other countries.

Surgical wound infections (SWI) are the most common complications in patients at the postoperative period. In the modern clinic, they worsen the prognosis of the disease, remain the most important and acute health problem in all countries of the world due to high prevalence, morbidity and mortality, as well as socio-economic damage [1–2].

Peculiarities of the pathogenesis of wound infections and types of their patients

Wound infection is a disease that causes pathogenic microorganisms to enter the human body through the wound and thus leads to the development of purulent and inflammatory processes.

Infection in the wound or infectious process develops when the balance between the microorganisms that contaminate the wound and the protective forces of the macroorganism, which is displayed as clinical symptoms of inflammation.

The mechanism of the wound process development is very complex and is still the subject of close attention and study by morphologists, microbiologists, immunologists, and clinicians. Its first period, defined as the melting of necrotic tissues and the cleansing of the wound defect from them, can be represented in the most general terms as follows. When natural external barriers (skin or mucous membrane) are damaged and microorganisms penetrate into the wound, the protective mechanisms of cellular (T-cells, polymorphonuclear leukocytes, macrophages) and humoral (B-cells) immunity come into action. At the same time, cellular immunity factors phagocytize microbial bodies and necrotic tissues, cleaning the wound. At this time, a granulation shaft is formed in the region of the edges of the wound, which prevents the spread of infection to the surrounding tissues. If the insufficiency of the body's defenses does not allow to reliably delimiting the wound, this can lead to a generalization of the infection [3–6]. Developing infectious complications are manifested in the form of near-wound abscesses, near-wound phlegmon, purulent streaks, fistulas, thrombophlebitis, lymphangitis and lymphadenitis. With generalization of infection, sepsis may develop [7].

During the infectious process in a wound, the pathogens spread through the blood and lymphatic system throughout the human body. After that, the acute phase of the inflammatory process begins, which occurs not only in areas of tissue damage, but also covers the whole body.

In the development of pathological processes, microorganisms are usually divided into saprophytes, opportunistic and pathogenic. One of the main criteria for the pathogenicity of microorganisms is invasiveness – the ability of microorganisms to multiply in the body, overcoming its defense mechanisms. Therefore, in the framework of this problem, it is believed that conditionally pathogenic bacteria, which are not characterized by an active form of invasiveness, can cause infections in wounds, in those clinical cases where anti-infective resistance is suppressed [8–9].

The generally accepted classification of wounds is their division into surgical and accidental. The first, in turn, are divided into "pure" and purulent. Accordingly, a certain pathogenic microflora is isolated in each type of wound [10–12].

"Clean" wounds occur during surgery in asepsis, where there is minimal exposure to bacterial microflora. The most common bacterial species detected were *Staphylococcus aureus* (37%), *Pseudomonas aeruginosa* (17%), *Proteus mirabilis* (10%), *Escherichia coli* (6%), and *Corynebacterium spp.* (5%). Polymicrobial infection was detected in 59 samples (27.1%) and was mainly of two types [13].

Staphylococci have a variety of antigens that are localized mainly in the cell wall: peptidoglycan and protein A, which is localized on its surface. It is *S. aureus* that has this protein, which is capable of non-specific association with Fc–fragments of IgG, which indicates the ability to agglutination with human serum and to a positive reaction when interacting with heterologous drugs [14–16].

Staphylococci induce large numbers of immunocytes, leading to inflammation and abscesses. Capsule polysaccharides inhibit the activity of phagocytes. Protein A, contained in the cell wall of *S. aureus*, has antiphagocytic properties. Pathogenic factors in bacteria of

the genus *Staphylococcus* are microcapsules, teichoic acid, protein A, as well as enzymes catalase, β-lactamase, lipase, hyaluronidase [10, 11, 17].

Purulent surgical wounds are most often infected with gram-negative microflora, mainly blue purulent bacillus (*Pseudomonas aeruginosa*) [18–19].

For a long time, *Pseudomonas aeruginosa* was considered an conditionally pathogenic microorganism, but due to the widespread use of antibiotics, the number of cases of various purulent-inflammatory processes caused by *P. aeruginosa* has increased significantly.

Pseudomonas aeruginosa is a gram-negative, rod-shaped bacterium that quickly adapts to different environmental conditions. As an obligate aerobic, it can also use anaerobic respiration using nitrates as electron acceptors [20].

O- and H-antigens are characteristic for *P. aeruginosa*. Pathogenic factors for this type of microorganism are exotoxin A, membrane toxins, lecocidin [21, 22].

The virulence of *Pseudomonas aeruginosa* is provided by saws, capsule shell, surface membrane proteins and cell wall, which are involved in adhesion processes. This microorganism produces a number of enzymes and toxins. The capsule-like glycoprotein is easily separated from the bacterial cell, provides protection against phagocytosis, and is toxic to host cells.

Diseases caused by this microorganism are primarily associated with purulent-inflammatory processes, which occur mainly in associations with staphylococcus. They are observed in the infection of surgical wounds and burns. Therefore, it is considered one of the main pathogens of NIS [10–12, 23].

Accidental wounds. This group of wounds includes traumatic wounds of various origins — domestic, industrial, gunshot wounds, etc. Such wounds are accompanied by significant damage and deep penetration into the body tissues of various foreign substances and particles. Accidental wounds are always primarily bacterially contaminated,

The main representative of the microflora of random infections are bacteria of the genus *Enterobacteriaceae — Escherichia coli* [24, 25].

This type of microorganism has a complex structure of antigens, which consists of somatic O-antigen, capsular K-actigen and flagellar — H-antigen.

In many cases, *E. coli* is the causative agent of exogenous purulent infections in various localizations of the body. It causes purulent processes together with bacteria of the *Staphylococcus* and *Pseudomonas* genus. Severe immunodeficiency can cause sepsis [17–22, 26].

Drugs of biological origin in the treatment of wound infections

Followed by the analysis of literature sources, it was found that in practice the fight against various types of wound infections is carried out comprehensively. The main drugs in the fight against pathogenic microflora are drugs of biotechnological origin, namely antibiotics, antiseptics for bandages and immunomodulators [1–2, 10, 27].

All strains of microorganisms that were removed from postoperative wounds showed polyresistance to most traditional antibiotics. According to official statistics, more than 30% of hospitalized patients receive antibiotics, of which almost half are patients for prophylactic purposes. Rational tactics of antibiotic therapy used for surgical type of wounds is pre-anesthetic administration of the first dose of antibiotic. Prophylactic antibiotics have been shown to reduce the incidence of postoperative complications from 40% to 2% in most cases.

Penicillin, aminoglycoside, fluoroquinolone, and cephalosporin antibiotics are the most commonly prescribed [28, 29].

The use of antibiotics is aimed at suppressing the microflora of the wound through the use of drugs with selective antimicrobial action. Topical administration of antibiotics and systemic antibacterial therapy, both in isolation and in combination with other agents, can effectively help to heal wound infection. However, the prerequisite for the choice of therapy is the selection of the dose and routes of administration [30, 31].

With a wide selection of antibiotics, the range of drugs for topical use is limited. After all, the use of local forms of antibiotics may be accompanied by an increase in the pathogen resistance. Also, the risk of developing resistance is significantly reduced if the drug for topical use is not used systematically. From this point of view, mucirocin and bacitracin are the most suitable drugs, because when using them there is no risk of selection of cross-resistance to other antimicrobial drugs.

Thus, antibiotics that are available in the form of ointments and powders for the local treatment of infected and purulent wounds include neomycin, bacitracin, fusidic acid, mupirocin, metronidazole, ofloxacin and others.

Examples of the above combinations that have shown high microbiological and clinical efficacy are Baneocin, a mixture of neomycin and bacitracin, and Neosporin, a mixture of bacitracin, neomycin and polymyxin B [29, 32–34].

However, the efficiency of application of existing antibiotics for the local treatment of wound infections is gradually declining, so the vectors of modern treatments are aimed at finding alternative ways to influence the wound process, one of which is the use of immunostimulatory drugs, including immunomodulators. different directions on the immune system depending on its initial state. They recover the normal functioning of the immune system in the required therapeutic doses, that is, they restore effective immune protection

For example, the use of recombinant IL-2, a means of local treatment of purulent wounds, mainly in combination with traditional treatments, causes an increase in lymphocytes and macrophages in the wound, thereby accelerating changes in the stages of the wound process, increasing the activity of phagocytic cells in the wound [35, 36].

Also promising is the use of a drug of local action — superlymph, which is a composite of a number of antimicrobial peptides and heterologous cytokines in their natural ratio in the fight against inflammatory diseases of different localization.

The study of the bacterial polysaccharides effect on neutrophilic granulocytes, which took place *in vitro* made it possible to establish their ability to affect not only the synthesis of cytokines but activate the main effector reactions of these cells as well, such as phagocytosis, chemotaxis, adhesion and inhibition of apoptosis. The ability of these immunomodulators to affect other cell populations, in particular monocytes and macrophages, has also been studied experimentally [37].

Due to the change in a body reactivity in the event of wound infections, new approaches are needed to diagnose disorders and their appropriate correction. Modern methods of immunodiagnostics enable to study even T- and B-systems of immunity, and this, in turn, made it possible to develop clinical and immunological criteria for assessing immune disorders in patients with wound infections, especially purulent, and monitoring the effectiveness of immunotherapy [35, 38–41].

Immunotherapy as one of the components of complex treatment of wound infections also gives positive results, especially the use of antistaphylococcal gamma globulin and hyperimmune antistaphylococcal plasma. The use of immunomodulators, especially thymic drugs, for this method of treatment is very promising [42–44].

As immunomodulators for wound healing in wound surgical infections, supernatants of adhesion-activated neutrophils are used, which are applied to the wound in 3–4 days after surgery, five times with an interval of 24 hours in 0.5–1.0 ml of diluted supernatant 3.0 ml of saline solution, and after 12 h apply bandages with antiseptics.

The use of supernatants can increase the intensity of therapy by stimulating local immunity. As a result: the local inflammatory reaction is decreased, the level of bacterial contamination of the wound is reduced and the rate of wound defect reduction is increased [45].

The principle of these immunomodulators use is the local application of autologous secretory products that are activated by neutrophil adhesion in addition to complex therapy for the treatment of purulent-inflammatory soft tissue diseases. An example of such a drug is Polyoxidonium – a drug with immunomodulatory action. It increases the body's resistance to various infectious diseases. The main mechanism of action is a direct effect on natural killers and phagocytic cells, as well as stimulation of appropriate antibodies formation. It is also characterized by detoxifying action, increases the resistance of cell membranes to cytotoxic substances, helps to restore immune responses in purulent-inflammatory processes and burns. It is used mainly in combination therapy [46, 47].

Interferons can be used as drugs with immunomodulatory action in the complex treatment of wound infections. For example, Interferon alfa 2b has an immunomodulatory, antiproliferative effect [48]. It contains recombinant human interferon that is completely identical to Interferon alpha 2b, which is synthesized by leukocytes of donor blood in response to interferon virus. It is non-toxic and harmless, especially effective in purulent-inflammatory processes that

accompany wound processes, is intravenous and endolymphatic administration of the drug [36, 49–53].

Conclusions

Thus, wound infections can be of different origins and are caused by different types of pathogens, among which the most common are microorganisms of the genus *Staphylococcus, Pseudomonas* and *Enterobacteriaceae*.

For the treatment of wound infections, regardless of its origin, in most cases a comprehensive therapy is used based on drugs of biotechnological origin, namely different types of antibiotics. Effective antibiotics are selected according to the type of pathogen and the damage degree to the body in order to determine the optimal course and dose of drugs.

Since the treatment of wound infections involves a comprehensive approach, the use of antibiotics, antiseptics for dressings and some immunomodulatory drugs is recommended.

After all, the latter are able to accelerate the healing process of wounds, by increasing the body's ability to carry out immune defense responses and by enhancing the effect of the main therapy.

Due to the fact that the use of immunomodulatory drugs in the treatment of this type of infection is still poorly understood and due to the high resistance of pathogens to existing traditional antibiotics, there is a large number of clinical studies in this area. The vector of their research is mainly aimed at finding individual drugs and complex therapies that will promote rapid wound healing, elimination of infectious contamination by possible pathogens and the use of a single therapy against polymicrobial type of wound infections.

This study did not receive any financial support from a government, community or commercial organization. The authors state that they have no conflict of interest.

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ХАРАКТЕРИСТИКА РАНОВИХ ІНФЕКЦІЙ ТА СПОСОБІВ ЇХ ЛІКУВАННЯ ІЗ ВИКОРИСТАННЯМ ПРЕПАРАТІВ БІОЛОГІЧНОГО ПОХОДЖЕННЯ

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Хірургічні ранові інфекції є найбільш частими ускладненнями у пацієнтів у післяопераційний період. У сучасній клініці вони погіршують прогноз хвороби, залишаються найбільш важливою і гострою проблемою охорони здоров'я в усіх країнах світу.

Метою роботи був аналіз сучасних наукових даних щодо особливостей патогенезу ранових інфекцій та типів їх збудників, а також препаратів біологічного походження у лікуванні ранових інфекцій.

У роботі детально обговорюється проблема інфікування ранових ушкоджень за оперативного втручання та побутових травмах різного характеру. Розглянуто основні збудники ранових інфекцій. Проаналізовано специфічні фактори патогенності для бактерій родів *Staphylococcus, Pseudomonas, Enterobacteriaceae*. На підставі аналізу джерел літератури, було визначено перелік препаратів біотехнологічного походження, що можуть ефективно використовуватися в комплексній терапії для лікування і профілактики ранових інфекцій.

Результатом роботи є виявлення тих механізмів патогенезу ранові інфекції, які обумовлюють ефективність використання препаратів біологічного походження у лікуванні даної патології.

Ключові слова: ранова інфекція, контамінація мікроорганізмами, *Staphylococcus, Pseudomonas, Enterobacteriaceae,* антибіотики, імуномодулятори.

UDC 16.853+57.086.83+616.089.843]:616.153.922-092.4
https://doi.org/10.15407/biotech15.02.015

USE OF KETOGENIC DIET THERAPY IN EPILEPSY WITH MITOCHONDRIAL DYSFUNCTION: A SYSTEMATIC AND CRITICAL REVIEW

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Received 10.01.2022
Revised 03.03.2022
Accepted 29.04.2022

Background. With the development of molecular techniques over time more than 60% of epilepsy has associated with mitochondrial (mt) dysfunction. Ketogenic diet (KD) has been used in the treatment of epilepsy since the 1920s.

Aim. To evaluate the evidence behind KD in mt dysfunction in epilepsy.

Methods. Databases PubMed, Google Scholar and MEDLINE were searched in an umbrella approach to 12 March 2021 in English. To identify relevant studies specific search strategies were devised for the following topics: (1) mitochondrial dysfunction (2) epilepsy (3) KD treatment.

Results. From 1794 papers, 36 articles were included in analysis: 16 (44.44%) preclinical studies, 11 (30.55%) case reports, 9 (25%) clinical studies. In all the preclinic studies, KD regulated the number of mt profiles, transcripts of metabolic enzymes and encoding mt proteins, protected the mice against to seizures and had an anticonvulsant mechanism. Case reports and clinical trials have reported patients with good results in seizure control and mt functions, although not all of them give good results as well as preclinical.

Conclusion. Healthcare institutions, researchers, neurologists, health promotion organizations, and dietitians should consider these results to improve KD programs and disease outcomes for mt dysfunction in epilepsy.

Key words: epilepsy; ketogenic diet; mitochondrial dysfunction; treatment.

Epilepsy is a neurological disorder characterized by epileptic seizures and unusual behavior that happens because of very harmful damage to neuronal cells in the brain especially in the lateral and temporal lobes [1]. According to the latest data of the World Health Organization (WHO), globally, an estimated five million people are diagnosed with epilepsy each year. In high-income countries, there are estimated to be 49 per 100 000 people diagnosed with epilepsy each year. In low- and middle-income countries, this can be as high as 139 per 100 000 [2].

Mitochondrial DNA (mtDNA) or genomic DNA mutations and mt dysfunction cause various diseases such as Lactic Acidosis, and Stroke-like Episodes (MELAS), Myoclonic Epilepsy and Ragged Red Fibers (MERRF), Progressive External Ophthalmoplegia, Leber Hereditary Optic Neuropathy, Kearns-Sayre Syndrome, and Leigh Syndrome (LS) etc. which can be diagnosed clinically [3–6]. In general, epileptic seizures are currently

controlled by nutrition therapies, antiepileptic drugs (AEDs) (Valproic acid, Carbamazepine, Phenobarbital, Phenytoin, Lamotrigine, Zonisamide, Topiramate and Levetiracetam), vagus nerve stimulations (VNS) and surgery [7, 8]. Although surgery seems to be the last solution in general, the most common treatment methods are AEDs and dietary approaches (i.e., ketogenic diet (KD) and Atkins Diet). KD is a high-fat, low-carb, and protein-rich diet and designed to mimic the anticonvulsant effects of fasting, which is known to suppress seizures [9]. Many studies have reported that KD is a safe, effective, and anti-seizure treatment for many types of epilepsy (especially for infantile epilepsies) [10, 11].

Relationship of mt Function, OS and Seizure

Epileptogenesis can be initiated by a range of brain lesions, including those due to tumors, infections, status epilepticus, childhood febrile seizures, stroke, hypoxia, traumatic brain injuries, and neurodegenerative diseases [5]. The frequency and severity of seizures occurring in the pathogenesis of epilepsy increases oxidative stress (OS) [4, 13–16]. AEDs are used in the clinic for seizure control in epilepsy due to mt dysfunction. However, some of AEDs (i.e., carbamazepine, phenobarbital, primidone, or oxcarbazepine) may cause OS by creating mt toxicity [17, 18]. Myoclonic and tonic seizures can coexist in situations that cause stress, as seen in Epilepsia Partialis Continua (EPC) [19, 20]. Status epilepticus is also well-known presentation feature of epilepsy with OS status in mt Respiratory Chain Complex (RCC) defects [21]. Mt dysfunction is related to epilepsy and can diagnosed by genetic or spectroscopic analyses, include neonatal epileptic encephalopathies with burst suppression due to mutation of the mt glutamate *SLC25A22* gene, neonatal onset epilepsy due to Lipoic Acid Synthetase *(LIAS)* deficiency associated with mutation c.7464A (p.Arg249His) in the *LIAS* gene, parieto-occipital epilepsy caused by a DNA Polymerase Subunit Gamma *(POLG) 1* gene compound heterozygous A467T/W748S genotype, intractable epilepsy due to *POLG* gene mutations, epilepsy with sensorineural hearing impairment, or diabetes mellitus due to a variant m.15218 A4G mutation, and Dravet Syndrome (DS) with comorbid gene mutations (c.3734 G4A and c. e733 C4T), and mt RCC I, II, III and IV dysfunction [8].

Effect of KD on mt Dysfunction and Brain Energy Metabolism

Neuronal cells under the effect of KD become more resistant to metabolic stress and epileptic seizure thresholds increase. Aggravation of seizure sensitivity increases the frequency and severity of seizures. KD is important in preventing OS that occurs in mitochondria due to increased ROS activity in neural cells. Ketone body (KB) are the metabolite of KD such as Acetoacetic Acid, beta-hydroxybutyric Acid and stimulates mt biogenesis, which reduces OS in mt and mtDNA mutation and improve mt function [12, 22]. KB reduces OS by clearing free radicals and increasing antioxidant levels [15]. Dutton et al. reported that KD reduced ROS activity in the mt of mice treated with 10–12 KD ($P < 0.05$) [23]. In addition, KD increased the endogenous antioxidant Glutathione (GSH) levels in mt, improved the mt redox state ($P < 0.01$), KD regulated GSH biosynthesis, increases the mt antioxidant status, protects mtDNA from oxidant-induced damage ($P < 0.05$) and provided seizure control [24, 25].

Direct neuronal effects induced by the KD may involve ATP-sensitive potassium (K_{ATP}) channel modulation, enhanced purinergic (i.e., adenosine) and Gamma Aminobutyric Acid (GABA)ergic neurotransmission, increased brain-derived neurotrophic factor expression consequent to glycolytic restriction, attenuation of neuroinflammation, as well as an expansion in bioenergetic reserves and stabilization of the neuronal membrane potential through improved mt function [27]. Glucose is the most preferred energy source in the brain in neuronal stimulation and sudden seizures. The KD's low carbohydrate content reduces the amount of glycolysis in the blood. KD mimics the metabolic state of starvation, forcing the body to utilize fat as its primary source of energy instead of carbohydrates. Glucose expenditure gets very high in neurons, but ATP deficit is provided by oxidation of KD during the epileptic seizures [28]. ATP is providing from KB that turn into the basic energy source for neurons by β-oxidation [26, 29]. KD increases the seizure threshold by increasing the amount of ATP with the change in glycolysis and mt function. Long-term KD therapy coordinates several genes involved in energy metabolism, provides increased energy stores such as mt biogenesis and phosphocreatine. This improves the function of neurons and causes fewer neurons to die under OS conditions [30].

KD also regulates neurotransmitter release due to energy use in neurons. Clanton et al. found that Glutamate is a mediator molecule between the neuronal and astrocytic compartments in the regulation of the GABAergic inhibiting tone. Glutamine synthetase deficiency is pathogenic process for the production of seizures both in the brain slice model and in the human neuropathological study [31–33]. In a contrast study, Chan et al. found that although KD treatment increased the GABA and agmatine levels but did not change glutamate levels in the hippocampus of rats [33]. Similarly, Calderón et al. found that KD has no effect on glutamate release [34]. Another study demonstrated that mt Dihydroorotate Dehydrogenase (DHODH) as a regulator of activity set points in hippocampal networks and DHODH inhibition is known to reduce susceptibility to seizures in the intractable epilepsy model [35]. KD can regulate to mt protein transcripts [36] so it may have a connection with DHODH. KD may help to inhibit DHODH and the seizures in epilepsy but there is no evidence.

An umbrella review approach was taken in recognition of the fact that a large body of literature exists on KD and epilepsy. The discovery of more than 270 identified mutations in mtDNA has further illuminated the clinical diagnosis of epilepsy and has been reported to continue to be a common feature in status epilepticus [12, 37] and previously published systematic reviews were only about epilepsy and KD outcomes and did not investigate KD effects on mt dysfunction in epilepsy groups, thus justifying the need for further research focusing on the changes that occur. This systematic review aimed to evaluate KD treatment on the mt dysfunction in epilepsy evidence with the following specific purposes: 1) to know the *in vitro and vivo*, clinical trials and case reports on mt dysfunction in epilepsy disease; 2) to verify some unanswered question about KD approach to mt dysfunction in epilepsy and 3) to evaluate the evidence of its validity and reproducibility.

Controversial Issues and Unanswered Questions

These questions were discussed with included studies in the following result sections. They can be important on the further research to be carried out in the future could lead to significant results (see Table 1).

Materials and Methods

Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) were followed as a method for reporting this article [38].

Literature Search and Study Selection

Three electronic databases (PubMed, Google Scholar, MEDLINE) were searched during the period of January 1980– 12 March 2021. The review focused on the KD treatment mechanism mt dysfunction in epilepsy. Besides, only articles published in English were considered. This review focused on topics KD treatment of mt dysfunction in epilepsy, specific search strategies were devised for the following topics: (1) mt dysfunction (2) epilepsy (3) KD. To achieve the maximum sensitivity of the search strategy, we combined the terms (Ketogenic Diet) OR (Ketogenic Treatment) OR (Ketone Bodies) AND (Mitochondrial Dysfunction) OR (Mitochondrial Mutation) AND (Epilepsy) OR (Seizure) OR (Epilepticus) OR (Mitochondrial Epilepsy) as keywords. Studies including mitochondrial disorders, mitochondrial dysfunctions in epilepsy disorders or in epilepsy model and the KD were considered eligible.

Results and Discussion

34 studies were found on PubMed, 1730 studies were found on Google Scholar, 30

Table 1. **Controversial issues and unanswered questions of KD treatment for mt dysfunction in epilepsy**

1. What is the scientific fact behind the preference of a KD in mt dysfunction in epilepsy?
2. Are there other specific treatments applied with/without KD for mt dysfunction in epilepsy?
3. Do KD and AEDs treatments really help mt dysfunction in epilepsy together?
4. Can KD Prevent drug resistance due to mt dysfunction in epilepsy?
5. What are the possible seizure prevention mechanisms of KD in epilepsy due to mt dysfunction?

studies were found on MEDLINE. In this study a total of 1794 publications were identified. A total of 36 publications were finally included. The flow chart of the literature search is presented in Fig. 1. Tables 2 to 6 summarized and contained the characteristics of the included studies.

Unanswered questions (in table 1) were tried to be answered with the included studies as following.

1. What is the scientific fact behind the preference of a KD in mt dysfunction in epilepsy?

Mt dysfunction in epilepsy is a potential clinic outcome, as mt OP provides the primary source of ATP in neurons and participates in the formation of ROS, which impacts neuronal excitability and synaptic transmission strongly [13]. A decrease in ATP and an increase in AMP, ADP, lactic acid, and an increase in intracellular Ca^{+2} [10] is indicators of dysfunction in mt [13] such as Alpers-Huttenlocher syndrome, Pyruvate Dehydrogenase Complex Deficiencies, LS, MELAS, MERFF, and *POLG*-related disorders which could present with focal generalized seizures, and this means that the patient is dealing with epilepsy caused by multiple mt mutations [39]. To diagnose mt dysfunction in epilepsy, there are some ways such as presence of epileptic seizures, multi-gene panel testing, many other mutation tests on the karyotype, the sequence of mtDNA and other mt causes (i.e., mt enzyme activity) [39, 40]. Looking at some examples from the included studies, Krysko and Sundaram reported that one of the common ND3 mt mutation T10191C can lead to epileptic seizures and high m.3243A> G heteroplasmy causes mt dysfunction in neurons so that, seizures can occur and the mt disorder can lead to epilepsy on the patient [40]. Homoplasmic

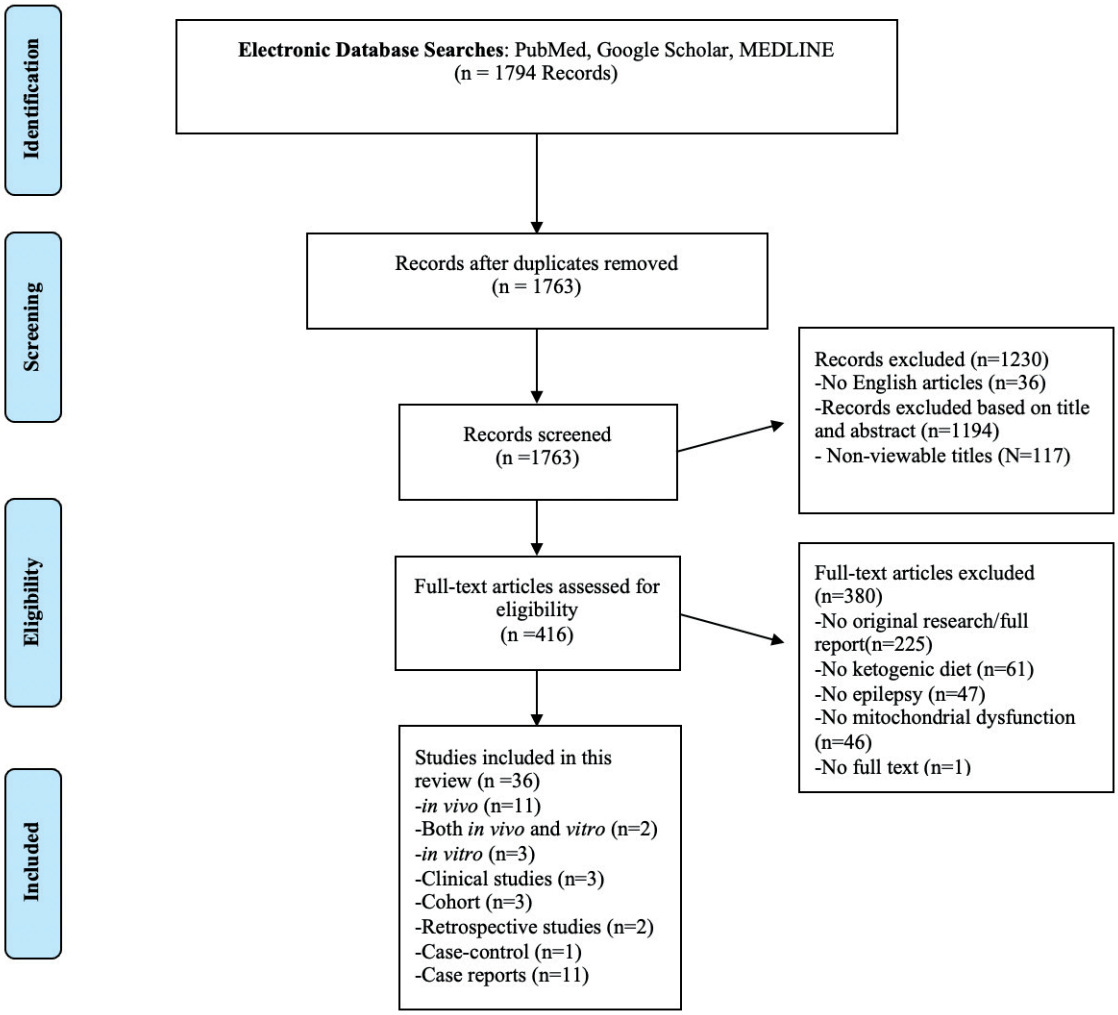

Fig. 1. **Summary of the process used to identify and select studies for the unanswered questions about the KD approach to mt epilepsy [38]**

and heteroplasmic mt mutations can cause mt dysfunction and the onset of various types of epilepsy [41]. Lee et al. had similar findings that 125 patients clinically suspected of LS and 25 patients were identified to have heteroplasmic mtDNA mutation associated to LS. Also, clinical features of this study can give some clues for mt dysfunction such as hyperlactatemia raised alanine, and muscle complex IV deficiency [42]. On the other hand, Gim nez-Cassina et al. reported that BCL-2-associated Cell Death Agonist (*BAD*) genetic variations that can be related to mt dysfunction because this genetic modification is very important to the prevention of apoptosis in mt and in the regulation of glucose and KB metabolism [43]. However, there was no other evidence, the effect of KD on AEDs response and seizures control in the studies regarding mt dysfunction in epilepsy caused by *BAD* mutation.

Twelve studies assessed the effect of KD on the mechanism of mt following the mutations or dysfunctions in epilepsy. Of the Twelve studies, only three were involved in the *in vitro* study. Geffroy et al. isolated *SH-SY5Y* human parental neuroblastoma cells from a postmortem patient with MELAS and containing 97.2% of the m.3243A> G. Then they treated cells for 3 weeks to explain the effect of KD for glucose restriction. As a result, they reported that OXPHOS protein and mtDNA copy number increased significantly and mt enzyme activity was improved in glucose restriction [44]. Frey et al. reported the same result by treated these cells (the same mutation) with KB for 4 weeks [45]. Hughes et al. also found the same results by applied the 250-μM C10 and C8 (on medium-chain triglyceride) diet to the *SH-SY5Y* neuronal cells separately [46].

Seven of the Twelve studies were related to *in vivo* studies. Nylen et al. applied the KD on *ALDH5A1*$^{-/-}$ mice (Succinic semialdehyde dehydrogenase (SSADH) deficiency) from postnatal 12th day and found that KD had very significant role to improve hippocampal mt and ATP levels [47]. Bough et al. evaluated the effects of KD by observing a significant increase in metabolic gene transcription, mt protein translation and biogenesis after a three-week administration of KD which may have an anticonvulsant effect in an animal model of epilepsy (Sprague–Dawley rats) due to pentylenetetrazole (PTZ), not a mt disorder [36]. Jarret et al. also measured mt redox activity of 3-week KD treatment in Sprague-Dawley rats (P28) and KD significantly improved ($P < 0.05$) mt functions by preventing mt oxidative damage [24]. Hasan-Olive et al. reported that the effect of KD on the *mutUNG1* transgenic mice was significantly higher in the pericaria and axon terminals of the hippocampus CA1 pyramidal neurons than Standard Diet (SD) fed mice. Subsequently, the hippocampal neurons obtained from rats were treated with β-hydroxybutyrate (βHB) and the Oxygen Consumption Rate (OCR) and the NAD+/NADH ratio improved significantly [48]. Wang et al. applied Autophagy Inducer Rapamycin (RAP) and inhibitor 3-methyladenine (3-MA) by manipulating autophagy after applying KD therapy in an epilepsy-induced rat model induced by PTZ. As a result, KD improved mt damage and reduced mt cytochrome c release, RAP pretreatment increased the neuroprotective effect of KD, and 3-MA pretreatment abolished it. In addition, KD increased the level of autophagy, and regulated pretreatment with RAP or 3-MA autophagy as well [49]. Kumar et al. reported that KD with *Scn1Lab* mutant zebrafish decreased in baseline glycolytic rate and OCR compared to controls and showed a significantly slower and exaggerated increase of both glycolytic rates and OCR after 4-aminopyridine. The significant result from this study, *Scn1Lab* mutant zebrafish suggest that glucose and mt hypometabolism contribute to the pathophysiology of DS which can be related with mt epilepsy [50]. Pérez-Liébana et al. studied KD treatment in Aralar/AGC1/Slc25a12 deficiency characterized by hypotonia, hypomyelination, developmental arrest and epilepsy. Five days βHB administration on Aralar-KO mice improved bad mitochondrial respiration by bypassing the metabolic failure in neurons. βHB improved DA, DOPAC/DA ratio, VMAT2 protein (dopaminergic system) and MBP, MAG myelin proteins (myelinization). Cytosolic aspartate and NAA synthesis increased with βHB oxidation in mitochondria. Aralar deficiency are probably eliminated through citrate-malate shuttle by increased cytosolic aspartate and NAA [74].

One study was related to case-report. Nizon et al. reported 2 ½-year-old French girl, a case who had focal seizures with Iron-Sulfur Cluster Scaffold (*NFU1*) mutation and Leukoencephalopathy. 6-month oral lipoid acid (100 mg/kg/day) was administered but was stopped because no improvement was observed in the disease. Subsequently, KD (lipids 60% of calories) was applied, but acute dystonia and metabolic attack were observed

within 24 hours, and it was reported that the reason was partially blocked Krebs cycle in the α-setoglutarate dehydrogenase step [51]. One study was related to clinical research (case-control study). Miles et al. reported 65 epilepsy patients with suspected mt dysfunction. No significant clinical, pathological or biochemical differences were found between the epilepsy and control groups. Only three patients treated with KD, 2/3 patients had valproic acid (VPA) and one of the patients had AEDs as well. All three patients had low subsarcolemmal mt aggregates (SSMA) but no evidence of increased mt density or improved ETC function has been shown in humans [18].

These included studies give us a wide range of explanations of the KD mechanism on mt function, but KD might have also different mechanisms and those should be revealed by supporting with further research. Three of *in vitro* studies showed that KD was useful for increasing OXPHOS protein, mtDNA copy number, mt enzyme activity and glucose restriction. Molecular diagnoses related to mt have been made in clinical and case studies published in epilepsy due to mt dysfunction. Except for the clinical follow-up of the patients in the KD and AEDs applied, molecular level investigations have not been reported. Overall, behind the general scientific reality that KD was effective in epilepsy due to mt problems, there were positive effects such as improved mt function, mt mass, mt biogenesis leads to improved alternative energy stores, restoring complex I, II, III and IV stability and activity, reduced mt cytochrome c release, increasing ATP synthesis, and decreasing the NADH/NAD+ ratio.

2. Are there other specific treatments applied with/without KD for mt dysfunction in epilepsy?

Of the seven studies assessing other specific treatments with/without KD on mt dysfunction in epilepsy. Willis et al. developed a new anticonvulsant diet (triheptanoin diet which contains high fat) in two types of chronic mouse epilepsy models. Triheptanoin feeding (high fatty acid) increased intracellular acylcarnitines which is very important for mt biochemical activity, decreased seizures and propionyl-CoA levels and increased methylmalonil-CoA level [52]. Hughes et al. reported medium-chain triglycerides feeding [application of decanoic acid (C10)] increased mt biogenesis significantly in Pharmaco-resistant epilepsy animal model *in vivo* and *SH-SY5Y* neuronal cells *in vitro*. This mt biogenesis might be related with the activation of the Peroxisome Proliferator Activator Receptor γ (PPARγ) [46]. Willes et al. applied KD and mt cocktail therapy during 3 months to Ohtahara Syndrome and reported that this combined therapy was useful for controlling seizures [52]. Similarly, Lee et al. reported 36/48 patients receiving the mt cocktail treatment showed an improvement as measured by the caretaker's global assessment form. Eight of these (16.7%) showed marked improvement. None of the cases had side effects. The clinical outcome from the mt cocktail supplement was not dependent on the type of mt Chain Enzyme Complexes (MRC) defect [20]. Seo et al. was used SD, 20% triheptanoin diet and 35% triheptanoin diet (high fat diet) for up to 7.5 weeks on CF1 mice model and high fat diet had an anticonvulsant effect to seizure control [53]. Cheng et al. also reported propionate and KD regulated mt dysfunction, neuron necrosis and epileptic seizures, reduced mt disruption, hippocampal apoptosis, neurological deficiencies, and epileptic seizure intensity [54]. Coenzyme Q10 supplements were useful to control seizures in epilepsy with mt dysfunction [39]. As a result, combined specific methods such as coenzyme Q10 supplements, mt cocktail therapy, triheptanoin diet, medium-chain triglycerides feeding, and propionate have anticonvulsant effect to seizure control for the epilepsy with mt function.

3. Do KD and AEDs Treatments really help mt dysfunction in epilepsy together?

65 percent of patients with epileptic seizures can be controlled with AEDs. However, despite the treatment and medication, 35 percent of patients with seizures are not still under control and these patients are known to develop resistant epilepsy [55]. According to studies, some AEDs can cause mt dysfunction (toxic with side effects) and the treatment process should be followed carefully [8, 13, 17]. Haj-Mirzaian et al. showed that the effect of mt disorder due to lipopolysaccharide on minocycline application was investigated minocycline reduces the seizure threshold and may reduce the side effects of AEDs through regulating of mt function and decreasing of neuro-inflammation in PTZ-induced seizures animal model [56].

Nine studies assessed mt dysfunction in epilepsy following KD treatment and AEDs. Nine of the seven studies were case reports. Wesoł-Kucharska et al. reported an 8-month infant male with LS (m.12706T > C in MTND5),

Table 2. ***In vitro*** **evidence of KD for mt dysfunction in epilepsy**

Authors	Type of Epilepsy	Cell Line	Aim	Treatment	Time	Results
Hughes et al. 2014 [46]	Pharmacoresistant epilepsy.	SH-SY5Y neuronal cell line.	Medium-chain triglyceride diet and the observation level of the plasma C8 and C10 concentrations.	250-μM C10 and C8.	6-days	C10 made an increase on mt number, citrate synthase along with complex I activity and catalase activity. C8 use revealed no significant effect with regards to citrate synthase activity. This may occur via the activation of the PPARγ.
Geffroy et al. 2018 [44]	MELAS Syndrome	SH-SY5Y parental neuroblastoma cell line (postmortem MELAS woman) and neuronal-like cybrid cells 97.2% m.3243A>G.	To evaluate the metabolic part of carbohydrate reduction in KD.	Low glucose exposure.	3 weeks	Accumulation of complex I matrix intermediates in untreated mutant cells, led to a severe reduction in complex I-guided respiration (parallels the postmortem brain tissue of a MELAS patient). OXPHOS protein coding and mt DNA copy number were significantly increased in mutant cells compared to other control fibroblast and neuron cells by glucose restriction (KD).
Frey et al. 2017 [45]	MELAS Syndrome	m.3243A>G with 98.6% mutant SH-SY5Y parental cell line	To investigate metabolic mechanisms of KB in MELAS syndrome.	KB exposure.	4 weeks	KB treatment restored complex I stability and activity, increased ATP synthesis, the mtDNA copy number and lowered the NADH / NAD+ ratio.

Abbreviations: Decanoic Acid (C10), Ketogenic Diet (KD), Ketone Bodies (KB), Lactic Acidosis and Strokes Like Episodes (MELAS), mitochondrial DNA (mtDNA), Octanoic Acid (C8), Oxidative Phosphorylation System (OXPHOS), Peroxisome Proliferator Activator Receptor γ (PPARγ), Quantitative Polymerase Chain Reaction (Q-PCR).

seizures and bad echocardiography. After administration of KD diet for 10-month clinical condition and echocardiography improved, after age 12-month AEI administration was used and successful result was received [75]. However, Buda et al. reported a 13 years old case diagnosed homoplasmic 8344G>A mutation in the Mitochondrially Encoded TRNA-Lys (AAA/G) (*MTTK*) gene with LS, MERRF and chronic epilepsy which treated with KD and VPA administration or intensive rehabilitation. The result of the benefit of KD after AEDs application for mt tRNA-mutated patients could not be assessed but was associated with remission [57]. Samanta et al. reported an 18-year-old case report with heterozygous *POLG* mutation-p.W748S (c.2243 G > C) (diagnosed with Intractable left-sided EPC) and several AEDs and KD therapy applied together, but no regression was observed in her seizures [58]. In contrast, Cardenas and Amato reported a 14-month-old female presented with EPC with *POLG* heterozygous mutations. Her seizures were eliminated but remained severely encephalopathic [59]. Krysko and Sundaram et al. reported a 16-year-old female case with MELAS (the rare *ND3* mt mutation *T10191C)* and primidone, phenytoin, topiramate, phenobarbital, perampanel were applied with the KD treatment, but her seizure frequency increased [40]. Kwong et al. similarly reported a 13-year-old Chinese boy and his family had *ARX* genetic defect (ARX-associated (c.989G>A; p.Arg330His) encephalopathy). His epilepsy failed to respond to various anticonvulsants including phenobarbital, clobazam, lamotrigine, levetiracetam, nitrazepam and KD treatment [60]. Similarly, Lankford et al.

Table 3. ***In vivo*** **and** ***in vitro*** **evidence for KD for mt dysfunction in epilepsy**

Authors	Type of Epilepsy	Animal Model	Aim	Treatment	Time	Results
1	2	3	4	5	6	7
Hasan-Olive et al. 2019 [48]	Refractory Epilepsy	WT mice and *mutUNG1* transgenic mice, rat hippocampal neurons	To test the effects of a KD or the βHB on induced mt toxicity in hippocampal tissue in vivo. To explore mechanisms underlying ketone increased mt biogenesis and functions *in vitro*.	In vivo: high fat KD In vitro: βHB exposure	–	that KD and βHB treatments apparently changed the expression of mRNAs and proteins *(UCP2, PGC1α, Drp1 and Mfn1)* and help to increase mt mass and functional competence, apparently via the "PGC1α-SIRT3-UCP2 axis ($p < 0.05$). ***in vitro*** side: Especially, βHB treatments increased OCR and the NAD+/NADH ratio. *in vivo* side: The mt count of *UCP2* was significantly higher in the perikaria and axon terminals of hippocampus CA1 pyramidal neurons in KD treated *mutUNG1* mice compared with *mutUNG1* mice fed a SD.
Giménez-Cassina et al. 2012 [43]	Epilepsy	$BAD^{-/-}$ and BAD^{S155A} knocking mice neuronal cells	To examine the role of *BAD* modifications on seizures by regulating KB and glucose metabolism.	KB exposure	-	*BAD* modification found to act mainly in the liver, brain cells and preventing mt apoptosis, increasing mt functions, regulating glucose metabolism, increase the function of K_{ATP} channel. Reduced seizures in increasing the use of KB metabolism. *BAD* modification was related to enhancing the functions of mt, increasing KB and glucose in direct proportion.
Willis et al. 2010 [52]	Epilepsy	CF1 mice	Anticonvulsant effects of feeding triheptanoin diet (high fat diet) (the triglyceride of anaplerotic heptanoate).	2%0 triheotanoin diet and 35% triheptanoin diet	7.5 weeks	35% Triheptanoin feeding increased intracellular acylcarnitines, decreased seizures and caused a decrease in propionyl-CoA levels and increased methylmalonil-CoA levels in SE mice. Intracellular acylcarnitines was in balance with fatty acid acyl-Coenyme A intermediates in mt fatty acid beta oxidation.
Dolce et al. 2018 [73]	Epilepsy	Male NIH Swiss mice (aged 3–4 weeks)	To find that the KD and intermittent fasting, would differ in their acute seizure test profiles and mt respiration.	1:4 KD or CD-IF (24 h feed/ 24 h fast)	12–13 days	KD protected the mice against 6 Hz-induced seizures but had more severe seizure scores in the kainic acid test and it was opposite in CF-IF. KD and CD-IF did not share identical antiseizure mechanisms. These differences were not explained by differences in mt respiration (significance was $P \leq 0.05$).

Table 3 (continued)

1	2	3	4	5	6	7
Bough et al. 2006 [36]	Epilepsy	Sprague–Dawley rats	Anticonvulsant effect of KD on epilepsy	KD	3 weeks	KD upregulated 34 transcripts of energy metabolic enzymes and transcripts encoding mt proteins, by increasing number of mt profiles. KD induced mt biogenesis, increased neuronal functions, metabolic gene expression and energy reserves. KD's anticonvulsant mechanism includes mt biogenesis leading to improved energy stores.
Jarret et al. 2008 [24]	Epilepsy	Adolescent Sprague-Dawley rats (P28)	KD effect on mt redox status in the adolescent rat brain.	KD	3 weeks	KD regulated GSH biosynthesis upwards, increased the mt antioxidant status, and protected mtDNA from oxidant-induced damage ($P < 0.05$).
Wang et al. 2018 [49]	Epilepsy	PTZ kindled rats	Protective role of autophagy activated by KD in brain injury following seizure and regulation of mt functions, particularly cytochrome c release, by autophagy by KD.	KD	4 weeks	KD reduced the mt cytochrome c release and improved mt damage and showed neuroprotective effect ($P < 0.05$). KD and pretreatment with RAP or 3-MA regulated autophagy ($P < 0.05$).
Kumar et al. 2016 [50]	DS (Several types of epilepsy)	*Scn1Lab* mutant zebrafish	To develop novel techniques of glycolysis and mt respiration in a zebrafish model.	KD	–	A decrease in baseline glycolytic rate and OCR, a significantly slower and exaggerated increase of both glycolytic rates and OCR after 4-AP. Five glycolytic genes found downregulated identified PCR array. *Scn1Lab* mutant zebrafish suggest that glucose and mt hypometabolism contribute to the pathophysiology of DS.
Fogle et al. 2016 [71]	Epilepsy- ME	Drosophila human ME ($ATP6^1$)	Investigate caloric restriction and KD against the seizures of ME, the K_{ATP} channel and therapeutic potentials on behavioral and neuronal level.	KD	-	KD is found highly effective at reducing seizures in the $ATP6^1$ model, improving time to recovery by up to 90% even in late-stage disease. High fat/KD benefits were dependent upon a functional K_{ATP} channel which was protective for seizures.
Fogle et al. 2019 [72]	Epilepsy- ME	Drosophila human ME ($ATP6^1$ and $TPI^{sugarkill}$ genotypes	To reveal KD mechanisms in the ME model, ketone bodies, citric acid cycle and anaplerotic supplements.	KD	-	Six of these eight drugs (carbamazepine, gabapentin, phenytoin, lamotrigine, topiramate, ethosuximide) had no significant effect on seizure. Vigabatrin and VPA made seizure recovery significantly.

Table 3 (end)

1	2	3	4	5	6	7
						KD was beneficial to multiple *Drosophila* seizure models including those caused by global energetic deficit due to mt dysfunction. KD can be useful on human glycolytic enzymopathy.
Stewart et al. 2008 [70]	SSADH deficiency	Adult *ALDH5A1*$^{-/-}$ mice with *C57/129S* and *WT*	To determine seizure detection method (movement velocity) and conducted a behavioral study of KD to characterize daily patterns of spontaneous motor seizures.	KD	From weaning day onward	KD exhibited a seizure phenotype characterized by fits of wild running clonus accompanied by jumping and bouncing. The seizure rhythm showed a peak shortly after the onset of the dark phase with periodicity close to 24 hours. Older wild type pups showed no evidence of abnormal motor behavior. Generalized tonic-clonic seizures are more frequent at a certain time of day in *ALDH5A1* $^{-/-}$ mice treated with KD.
Pérez-Lie̬bana et al. 2020 [74]	Aralar/AGC1/Sl-c25a12 deficiency	*Aralar-KO*	Effect of βHB, in neuroprotective in Aralar-KO neurons and mice (AGC1-deficiency is characterized by hypotonia, hypomyelination, developmental arrest and epilepsy).	Injections of βHB	5 days	Dopaminergic system improved (DA, DOPAC/DA ratio and VMAT2 protein). MBP and MAG myelin proteins were markedly increased in the cortices. Increase in aspartate (3-fold) and NAA (4- fold) levels. It improved imperfect mitochondrial respiration by bypassing the metabolic failure in neurons. βHB oxidation in mitochondria increases the synthesis of cytosolic aspartate and NAA.

Abbreviations: 4-aminopyridine (4-AP), Aldehyde Dehydrogenase 5 Family Member A1 (ALDH5A1), Antiepileptic Drug (AED), BCL-2-associated Cell Death Agonist (BAD), Control Diet (CD), DNA Repair Enzyme UNG1 (mutUNG1), Dietary Modifications (DMs), Dopamine (DA), Dravet Syndrome (DS), Drosophila model of human ME (ATP61), Fission Protein Dynamin-related Protein-1 (Drp-1), Glutathione (GSH), Inhibitor 3-methyladenine (3-MA), Ketone Bodies (KB), Ketogenic Diet (KD), Micronutrients with Intermittent Fasting (CD-IF), Mitofusin-1 (Mfn-1), mt Uncoupling Protein-2 (UCP2), mt Encephalomyopathy (ME), Mitochondria (mt), models of human glycolytic enzymopathy (TPIsugarkill), Normal Diet (ND), Oxygen Consumption Rate (OCR), Oxidized Glutathione (GSSG), Oxygen Consumption Rate (OCR), Peroxisome Proliferator-activated Receptor-γ Coactivator-1α (PGC-1α),, Pentylenetetrazole (PTZ), Autophagy Inducer Rapamycin (RAP), Standard Diet (SD), Status Epilepticus (SE), Succinic Semialdehyde Dehydrogenase (SSADH), Succinic Semialdehyde Dehydrogenase Deficiency (SSADH-d), Mutations in a Voltage-activated Sodium Channel, Nav1.1 (Scn1Lab), Valproic acid (VPA), Wild Type (WT), β-hydroxybutyrate (βHB).

reported 6-year-old girl case with diagnosed with a complex I–deficient mt disorder and *SLCA2* gene mutation. She did not respond to drug treatment. KD was added to topiramate, levetiracetam and ethosuximide for two days but never continued as was seizures persisted [61]. Of the two study were clinical trials. Lee, Na and Lee examined that 25/125 patients were identified to have mtDNA mutation with LS and KD was given to 14 patients and 2 of them did not respond AEDs treatment [42]. Additionally, Saneto et al. reported 2/180 of children and adolescents with mt disease and they took KD and two traditional AEDs. However, the results of this combined treatment have not been reported [62].

Most of the studies included showed that KD and AEDs treatments did not respond well in epilepsy cases with mt disorders. KD is known to provide energy and reduces ROS and cure mt disorders [50] but according to included studies, KD did not have a positive effect on mt dysfunction in epilepsy. It may mean that the results of the combined administration of KD and AEDs therapy may vary with mt dysfunction with multiple mutations. In preclinical studies, KD were quite successful on mt functions. On the other hand, some cases can indicate that the use of KD with AEDs do not affect patient's clinical patterns. What may be the difference between the studies modeled in a laboratory environment from human studies? Are scientists failing to consider comorbid conditions that occur in patients with mt disorders? Consequently, the underlying failure of clinical trials and case-reports involving KD, and AEDs therapy can be supported by *in vitro* studies on samples as well to be collected from patients, supported by future research into mt function.

4. Can KD prevent drug resistance due to mt dysfunction in epilepsy?

According to included studies there were only two study related to KD prevention on AEDs resistance. Lee, Na and Lee reported 25/125 patients were identified to have mtDNA mutation with LS and AEDs response failure. KD was given to 14/25 patients and only 2 of them did not respond AEDs treatment [42]. Lee et al. reported 24 out of 48 epileptic children with medically intractable epilepsy were treated with a KD. However, there is no report regarding the drug response of KD. The important point here was that mt respiratory MRC defects progressed with the diagnosis of epilepsy due to many mt dysfunction clinically. In this study, depending on the MRC defects (in Table 4), there were also two cases (4.2%) of Ohtahara Syndrome, 10 cases (20.8%) of West syndrome, 12 cases (25.0%) of LGS, two cases (4.2%) of Landau-Kleffner syndrome, 14 cases (29.2%) of unclassified generalized epilepsy, and eight cases (16.7%) of partial epilepsy have been reported [53]. In conclusion, the treatment of KD needs further investigation in epilepsy models *(in vivo and vitro)* and clinics that have both mt dysfunction and drug resistance. There is no such evidence in the literature from a preclinical point of view and only one clinical study has been reported for this question.

5. What are the Possible Seizure Prevention Mechanisms of KD in Epilepsy due to mt Dysfunction?

There were twenty-one studies looked at prevention of KD treatment against of seizures in mt dysfunction in epilepsy. Six of twenty-one studies were clinical reports. Amin et al. indicated that forty-four patients with *CDKL5* mutation were taken AEDs, VNS and KD treatments. Twelve patients received VNS treatment, but six patients had epileptic seizure control. After one year of VNS treatment, this number increased to nine patients in seizure control. Subsequently, twenty-six patients received KD treatment, only twelve patients had a positive turn and sixteen patients had serious side effects. Epileptic seizures of 40/42 patients who only took AEDs could not be controlled at the end [63]. Na et al. reported a retrospective study about Lennox-Gastaut syndrome [(LGS) a typical intractable form of epilepsy] patients with mt dysfunction and they had diet therapies (DT) [(16/20 patients had KD therapy)] and KD therapy was found to be beneficial and significantly improve patient's prognosis in seizure control [64]. Lee et al. presented another retrospective study about 40/372 LGS patients with mt dysfunction and they were classified into two groups based on the history of West Syndrome (WS) (total 13 WS and 27 No WS patients). The initial symptoms of the patients were seizure (50%), delayed development (40%), ataxia, hemiparesis, perinatal asphyxia, and loss of consciousness. The results of KD treatment were shown in table 4. However, this study also did not report definitive results for the effects of KD, and it was reported that there was limited to not continuing to determine a prospective random model in selecting the subjects and determining other research items [65]. Lee et al. reported 48 epileptic patients (23 male, 25 female) with MRC defects (ratio of the MRCs defects were presented in table 4). 18 children (75.0%) with medically intractable epilepsy were treated with a KD, over 50% demonstrated a decrease in seizure frequency and 50.0% even became seizure-free [20]. Saneto et al. reported 174/180 children and adolescents with mt disease, 85 of them had seizures and 21 of them tried to take KD treatment. Seven of the 21 patients on the KD responded positively with over 75% seizure reduction. One patient was diagnosed with Pyruvate Dehydrogenase Complex Deficiency and was on the KD until death. This patient was not completely seizure-free, but breakthrough seizures were infrequent,

Table 4. **Clinical outcomes of KD for mt dysfunction in epilepsy**

Author	Type of Epilepsy	Type of Study	Aim	Patients	Treatment	Time	Results
Na, Kim and Lee 2020 [64]	LGS (a typical intractable form of epilepsy)	Retrospective	To investigate the clinical efficacy and safety of KD	20 LGS patients with mt dysfunction	DTs	2004–2014	16 patients received a KD with a lipid:nonlipid ratio of 4:1. 2 patients received a lipid:nonlipid ratio of 3:1. KD showed which efficacious and feasible for LGS patients with mt dysfunction and can significantly improve their prognosis. Improvement of seizures and cognitive function were not inferior to those in other patients treated with KD.
Lee, Baek and Lee 2019 [65]	LGS (a typical intractable form of epilepsy)	Retrospective	To investigate the clinical manifestations, diagnoses, treatments, and epilepsy in LGS	372 patients mt disease between	KD	2006–2016	*KD Retention with West Syndrome:* Nine out of 13 patients received KD, but 7 patients received more than 6 months and 2 patients less than 6 months. *KD Retention with no West Syndrome:* Of the 27 patients, 15 patients received KD, but 7 patients took longer than 6 months and 8 patients received less than 6 months.
Lee et al. 2008 [20]	Childhood epilepsy, intractable epilepsy	Clinical	To demonstrate that MRC defects in children with epilepsy	48 epileptic patients (23 male, 25 female) with MRC defects	KD, mt cocktail with coenzyme Q10 (5 mg/kg/day), vitamin B and C (25 mg/kg/day), vitamin E (200–400 mg) and L-carnitine (100 mg/kg/day)	–	MRC I deficient was 35 patients. MRC II deficient was 1 patient. No MRC III deficient reported. MRC IV deficient was 11 patients. MRC I+IV deficient together were 1 patient. *KD produced clinical improvements, including seizure reduction and global functional improvement in over 50% patients.*
Lee, Na and Lee 2019 [42]	Leigh Syndrome-Epilepsy	Clinical	To describe its dominant neurological clinical features and analyze data related to epilepsy in Leigh syndrome accompanied by a mtDNA mutation.	125 patients, 25 patients were identified to have mtDNA associated Leigh syndrome	KD	–	Patients with epilepsy in Leigh syndrome. There were 14/2 patients who was on KD and did not respond to anti-epileptic drugs.
Amin et al. 2017 [63]	Childhood epilepsy (drug-resistant epilepsy)	Cohort Study	To investigate the efficacy of different treatment modalities for epilepsy.	44 patients with *CDKL5* mutation (Thirty-nine female and Five male).	AED, VNS and KD		27 patients received a KD during two years for seizure control. 12 of them had positive results. Side effects: constipation, vomiting and feeling tired were observed in 18/26 patients. Half of the patients experienced improved quality of life with KD.

Table 4 (end)

1	2	3	4	5	6	7	8
Saneto et al. 2017 [62]	Multi-regional epileptiform-status epilepticus	Cohort study	To describe the age of onset, EEG, seizure semiologist, response to medical management, and outcomes in a large cohort of infants, children, and adolescents with mt disease.	180 patients	AED and KD		7/21 patients on the KD had a positive response over 75% for the seizure reduction. 1 patient diagnosed with pyruvate dehydrogenase complex deficiency and was on the KD until death. This patient was not completely seizure-free, seizures were infrequent, 2 to 3 per month. 3 patients seizure-free, but had to be removed from the diet for compliance or quality of life issues. 2 patients who remained on the diet remained seizure-free to date, as stated above, the other 2 had seizures, but a certain decrease in seizure frequency was observed and a positive return was understood. 6 patients with epileptic spasms tried the KD and only 2 patients could go on the diet. 1 patient with complex III dysfunction became seizure-free for 5 years on the KD but had to be discontinued.
Vasta et al. 2012 [66]	Generalized epilepsy with febrile seizures plus type 2 DS	Cohort study	To analyze 26 patients with known or highly suspected mt disease of 908 nuclear genes and validate the methodology	18 patients	KD	–	Complex III deficiency in genetic tests: I / III = 31%, I = 59%, II / III = 47%, II = 89%, III = 16%, IV = 47%, CS = 107%. Also, the SCN1A mutation, C> T3733; Used for reference control found (SCN1A [NM_001165963], c.3733C> CT p.Arg1245Term, 604233/607208 / dominant). This mutation causes febrile seizures plus type 2/DS and generalized epilepsy. Seizures became overwhelming with multiple drugs, but the KD improved dramatically in motor skills and language development.
Miles et al. 2012 [18]	Epilepsy	Case-Control Study	To evaluate the effects of epilepsy-related factors associated with mt disorders.	65	VPA, KD, AED	2000–2008	No significant clinical, pathological, or biochemical differences were found between the epilepsy and control groups. 3 patients on the KD had low SSMA i.e., 0%, 0.5%, and 5%. The average SSMA for the epilepsy and control groups was 6.1% and 7.7%, respectively. The possibility of a KD to increase mt density or ETC complex activities could not be conclusively influenced in this study.

Abbreviations: Antiepileptic Drug (AED), Burrows-Wheeler Aligner (BWA), Cyclin-dependent Kinase-like 5 (CDKL5), Carnitine Palmitoyltransferase Type 2 Gene (CTP2), Dravet Syndrome (DS), Diet Therapies (DT), Electroencephalography (EEG), Electron Transport Chain (ETC), Ketogenic Diet (KD), Lennox-Gastaut Syndrome (LGS), Mitochondria (mt,) Mitochondrial Respiratory Chain Enzyme Complexes (MRCs), Diphosphate Synthase, Subunit 1 (PDSS1), Polymerase Gamma 1 (POLG1), Respiratory Chain Complex (RCC), Sodium Voltage-Gated Channel Alpha Subunit 1(SCAN1A), Subsarcolemmal Mitochondrial Aggregates (SSMA), Subunit D (SDHD), Ubiquitin-protein Ligase E3A (UBE3A), Vagal Nerve Stimulator (VNS), Valproic Acid (VPA).

2 to 3 per month. Three of the respondents were seizure-free but had to be removed from the KD for compliance or quality of life issues. While two of the patients who remained on the KD remained seizure-free to date, as stated above, the other 2 had seizures, but a certain decrease in seizure frequency was observed. Six patients with epileptic spasms tried the KD and only 2 patients could go on the diet. On the other hand, one patient with complex III dysfunction, became seizure-free for 5 years on the KD, but had to be discontinued due to family problems [37]. Vasta et al. performed genome analysis of 26 patients with mt disease and reported a young child who had hypotonia, language delay, delayed milestones, and ataxia with febrile seizures clinically. The patient had Complex III deficiency and multiple mutation which were characterized by seizures. Seizures became overwhelming with AEDs, but the KD treatment improved surprisingly her motor skills and language development [66].

Of eight studies were case reports. Ait- El-Mkadem et al. observed three case reports, it was conducted positive effects with KD which given to patients with mt Malate Dehydrogenase (MDH) 2 encodes mt MDH and refractory epilepsy showed reduction seizure frequency [67]. Seo et al. reported a case report with Ohtahara syndrome (intractable epileptic syndrome) with MRC I defect. KD and mt cocktail therapy showed completely controlled seizures and suppression burs patterns disappeared [23] also Cárdenas and Amato reported that a patient with Alpers-Huttenlocher Syndrome (diagnosed *POLG* heterozygous mutation), seizures were completely eliminated in the combined application of KD and AEDs therapies [59]. Similarly, Joshi et al. reported a case Alpers-Huttenlocher Syndrome (diagnosed two *POLG* heterozygous mutation). According to the result, the treatment with KD improved the patient's clinical prognosis and electroencephalogram (EEG) results [68]. Steriade et al. reported 22-year-old woman with multiple episodes of generalized and focal status epilepticus and migratory cortical stroke-like lesions (mtDNA disease-causing (m.3260A>G) transition mutation in the Mitochondrially Encoded tRNA leucine 1 (UUA/G) (*MT-TL1)* at 85% mutant heteroplasmy and after one-year KD treatment, she had continued to remain seizure free with no further stroke-like episodes [69]. In contrast, Krysko and Sundaram et al. reported patient with complex IV deficiency indicated that she had mt dysfunction and she treated with some multiple AEDs and KD, unfortunately, seizures have increased gradually [40]. Similarly, Samanta et al. reported a case who had heterozygote *POLG* mutation with mt dysfunction and treated with KD and some AEDs, likewise no improvement on his seizures [58]. The same seizure failure has been reported by Lankford et al. (see in Table 5) [61].

In the preclinical side there were seven studies. Stewart et al. showed that KD treatment was effective to control tonic-clonic seizures on *Aldh5a1 null (Aldh5a1*$^{-/-}$*)* mice model [70]. *Similarly,* Fogle et al. found that KD was more beneficial to AEDs but depended on K_{ATP} channel function which should be supported by AEDs to reduce seizures on *Drosophila ATP6*^{1} model of mt encephalomyopathy previously [71]. After that, the same group showed two drugs (VPA and Vigabatrin) reduced seizures on the same model from eight AEDs and pharmacological therapy could be much more effective with KD for reducing seizures [72]. Giménez-Cassina et al. showed the *BAD* gene can also be effective in KB metabolism and associated with epileptic seizure *in vivo* and *vitro*. *BAD* modifications resulted in a significant increase in the activity of metabolically sensitive K_{ATP} channels in neurons, as well as in resistance to behavioral and electrographic seizures in *Bad*$^{-I-}$ and *Bad*^{S155A} knocking mice [43]. Additionally, Kumar et al. presented the effect of KD application on seizures in epilepsies due to mt damage in *Scn1Lab* mutant zebrafish. The neuroprotective effect of KD application against epileptic seizures was revealed by increasing mt cytochrome c release ($P < 0.05$) [19]. Willis et al. also found that 35% Triheptanoin feeding (high fatty acid) decreased seizures in CF1 mice model [52]. Dolce et al. reported KD treatment protected the Male NIH Swiss mice against to 6 Hz-induced seizures but had more severe seizure scores in the kainic acid test. This study also compared to micronutrients with intermittent fasting (CF-IF) and KD, as a result CD-IF did not share identical antiseizure mechanisms as a KD treatment [73].

Consequently, on average, more than 50% of the clinical trials with KD contributed to seizure control. While 5 cases out of 8 case studies showed positive effects in seizure control, only 3 cases did not show positive effects. Included preclinical studies also found that KD was effective on seizure control in epilepsy models with mt disorders.

Table 5. **Case report outcomes of KD for mt dysfunction in epilepsy**

Author	Type of Epilepsy	Type of Study	Aim	Patient	Treatment	Time	Results
Joshi et al 2009 [68]	Alpers-Huttenlocher Syndrome- Epilepsia partialia continua	Case report	To report a patient with Alpers-Huttenlocher Syndrome (mt depletion syndrome).	Infant female	KD	–	Sequencing of the mt *POLG* gene revealed two heterozygous mutations. First mutation, c.2243G>C, and the second was a novel splice donor-site mutation, c.2480+1g>a. *Improved clinically, and EEG improved after KD.*
Cardenas and amato 2010 [59]	Alpers-Huttenlocher Syndrome — Status epilepticus	Case report	To report a patient with Alpers-Huttenlocher Syndrome (mt depletion syndrome).	14-month-old female	AED and KD	–	c911T > G (p. L30 4R) mutation was accompanied by an unknown mutation c.1174C>G (PL39 2V) and a 3240-3242 duplication (pR1081dup). *Multiple AEDs with KD eliminated her seizures, but she remained severely encephalopathic.*
Seo et al. 2010 [53]	OS (intractable epileptic syndrome)	Case report	To report a patient with OS that is associated with MRC I defect.	3month age, OS patient	KD and mt cocktail therapy	–	With KD and mt cocktail therapy, seizures were completely controlled, and suppression-burst patterns disappeared 3 months after starting treatment.
Buda et al. 2013 [57]	MILS, MERRF Epilepsy	Case report	To report child with mt disorder (m.8344G>A mutation)	13 years old child	KD	–	VPA administration or intensive rehabilitation associated with worsening. The benefit of a KD for mt tRNA-mutated patients cannot be assessed until prospective blinded studies in larger groups of patients are performed. KD associated with remission.
Ait-El-Mkadem et al. 2017 [67]	Refractory epilepsy	Case report	To report clinical and genetic findings if 3 patients who have Biallelic *MDH2* Variants. Note: MDH2 encodes MDH.	P1: partial, afterward myoclonic P2: generalized tonic and spasms P3: myoclonic epilepsy and generalized tonic	P1: KD P2: KD P3: KD	P1: 3-year P2:18month P3: 2 years	P1: reduction epileptic seizure frequency alive in 5 years P2: reduction epileptic seizure frequency but died after 1.5 years (secondary to metabolic decompe Demonstration of all *in vitro* results related KD use in mt dysfunction in epilepsy nsation) P3: ? alive at 12 years.
Krysko and Sundaram 2018 [40]	Myoclonic epilepsy due to MELAS with the rare *ND3* mt mutation *T10191C*.	Case report	To report patient with myoclonic epilepsy	16-year-old female patient	KD	–	Seizure frequency increased despite the addition of primidone, phenytoin, topiramate, phenobarbital, perampanel, and the KD. Entered terminal refractory status epilepticus despite appropriate optimization of AEDs and mitochondrial supplementation.

Table 5 (continued)

1	2	3	4	5	6	7	8
Kwong et al.2019 [60]	Infantile epileptic-dyskinetic encephalopathy and clarified the unknown genetic etiology	Case report	To report and detect if *ARX* genetic defect is associated with a spectrum of neurodevelopmental disorders.	13-year-old male and his family	AEDs and KD	–	His epilepsy failed to respond to various anticonvulsants including phenobarbital, clobazam, lamotrigine, levetiracetam, nitrazepam and KD. ARX-associated (c.989G>A; p.Arg330His) encephalopathy showing mitochondrial dysfunction and transient responsiveness to pyridoxal phosphate treatment.
Steriade et al. 2014 [69]	MELAS	Case report	To report patient with multiple episodes of generalized and focal status epilepticus and migratory cortical stroke-like lesions.	22-year-old woman	KD	1 year	Muscle biopsy showed a mitochondrial DNA disease-causing (m.3260A>G) transition mutation in the *MT-TL1* gene at 85% mutant heteroplasmy. Skeletal muscle biopsy has 38% heteroplasmy for the same m.3260 A>G transition mutation. Her mother was asymptomatic and had almost 5% heteroplasmy for m.3260A>G in blood derived mitochondrial DNA. KD application for one year made her seizure free with no further stroke-like episodes.
Samanta et al 2019 [58]	Intractable left-sided EPC	Case report	To report patient with an EPC and mitochondrial dysfunction	18-year-old male *POLG* p.W748S, p.S305R with mutations			The patient was treated with several AEDs such as midazolam infusion, fosphenytoin, levetiracetam, lacosamide, pentobarbital infusion, clonazepam, topiramate, VPA and KD but *no definite improvement in his frequency of seizures*
Nizon et al. 2014 [51]	Secondary lesional epilepsy	Case report	To report patient with *NFU1, PDHA1* mutations and leukoencephalopathy with focal seizures.	2 ½ -year-old female	lipoic acid (100 mg/kg/day) then KD (lipids 60% of calories)	6 months	*NFU1* mutation and a generalized mt complex deficiency predominant on complex II were revealed. Lipoic acid did not prevent neurological regression without any effect and stopped after six months. However, trial of KD has worsened the patients status. She presented an acute episode of dystonia and metabolic attack within 24 hours following the test. *Possibly it happened because of the partial block of the Krebs cycle at the α-cetoglutarate dehydrogenase step.*
Lankford et al. 2011 [61]	Intractable epilepsy (multiple seizures per hour)	Case report	To report patient with multiple seizures.	6-year-old female	AEDs, DT	–	Spectrophotometric analysis demonstrated an absence of complex I activity. Diagnosed with complex I–deficient mt disorder and a mutation in *SLCA2* gene. *She failed therapy with topiramate, levetiracetam, and ethosuximide. KD was given for 2 days but never continued as was seizures persisted.*

Table 5 (end)

1	2	3	4	5	6	7	8
Wesoł-Kucharska et al. 2021 [75]	LS (m.12706T>C in MTND5), seizure	Case report	To report a LS patient	Male 8-month infant	KD	10 months	After 10-month echocardiography and general condition improved. Although after age of 12-month patient required implementation of treatment with AEDs.

Abbreviations: Anti-epileptic Drugs (AEDs), Aristaless-related Homeobox (ARX), Diet Therapies (DT), electroencephalography (EEG), Epilepsia Partialis Continua (EPC), Ketogenic Diet (KD), Leigh Syndrome (LS), Magnetic Resonance Imaging (MRI), Mitochondrial Encephalopathy with Lactic Acidosis and Stroke-like Episodes (MELAS), Mitochondria (mt), Mitochondrial Respiratory Chain Enzyme Complexes (MRCs), Myoclonic Epilepsy with Ragged Red Fibres (MERRF), Mitochondrially Encoded tRNA Leucine 1 (UUA/G) (MT-TL), Mitochondrially Inherited Leigh Syndrome (MILS), Mitochondrial Malate Dehydrogenase (MDH), Mitochondrial Malate Dehydrogenase 2 (MDH 2), Mitochondrially Encoded TRNA-Lys (AAA/G) (MTTK), Mitochondrially Encoded tRNA leucine 1 (UUA/G) (MT-L1) Iron-Sulfur Cluster Scaffold (NFU1), Ohtahara syndrome (OS), Pyruvate Dehydrogenase E1 alpha 1 subunit (PDHA1), Polymerase Gamma 1 (POLG), Solute Carrier Family 2 (SLCA2), Facilitated Glucose Transporter Member 1 Gene (SLCA2), Valproic Acid (VPA).

To the best of our knowledge this is the first report systematically appraising KD treatment for the mt dysfunction in epilepsy. The systematic review process identified 36 articles which met the inclusion and exclusion criteria. This review discusses the topic starting from the basics with five questions with the included preclinical, clinical, retrospective and case studies. This umbrella review makes a number of recommendations for KD applications to preclinical and clinical practice in mt epilepsies for the future research. Healthcare institutions, researchers, neurologist, health promotion organizations and dietitians should consider these in order to improve the effectiveness of KD interventions for patients with multiple mt dysfunctions in epilepsy. Healthcare settings, researchers, neurologist, health promotion organizations, dietitians and neuropsychiatry should consider these in order to improve the effectiveness of KD interventions for patients with multiple mt dysfunctions in epilepsy.

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ВИКОРИСТАННЯ КЕТОГЕННОЇ ДІЄТЕРАПІЇ В ЕПІЛЕПСІЇ З МІТОХОНДРІЙНОЮ ДИСФУНКЦІЄЮ: СИСТЕМАТИЧНИЙ ТА КРИТИЧНИЙ ОГЛЯД

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З розвитком молекулярних методів з часом більше 60% епілепсії асоціювалося з мітохондріальною (мт) дисфункцією. Кетогенна дієта (КД) використовується для лікування епілепсії з 1920-х років.

Мета. Оцінити докази, що лежать в основі дисфункції МТ при епілепсії.

Методи. У базах даних PubMed, Google Scholar та MEDLINE було здійснено загальний підхід до 12 березня 2021 року англійською мовою. Для визначення відповідних досліджень були розроблені конкретні стратегії пошуку за такими темами: (1) мітохондріальна дисфункція, (2) епілепсія, (3) лікування КБ.

Результати. З 1794 статей до аналізу було включено 36 статей: 16 (44,44%) доклінічних досліджень, 11 (30,55%) повідомлень про ипадки, 9 (25%) клінічних досліджень. У всіх доклінічних дослідженнях KD регулював кількість профілів mt, транскриптів метаболічних ферментів та кодувальних протеїнів mt, захищав мишей від судом і мав протисудомний механізм. Звіти про випадки та клінічні випробування повідомляли про пацієнтів з хорошими результатами в контролі судом та функціях МТ, хоча не всі вони дають хороші результати, а також доклінічні.

Висновок. Закладам охорони здоров'я, дослідникам, невропатологам, організаціям зі зміцнення здоров'я та дієтологам слід урахувати ці результати, щоби покращити програми КД та результати захворювання при дисфункції МТ при епілепсії.

Ключові слова: епілепсія; кетогенна дієта; дисфункція мітохондрій; лікування.

EXPERIMENTAL ARTICLES

UDC 57.089.6:571.27 https://doi.org/10.15407/biotech15.02.037

LONG-TERM EFFECTS OF SHAM SURGERY ON PHAGOCYTE FUNCTIONS IN RATS

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Received 02.02.2022
Revised 19.04.2022
Accepted 29.04.2022

Animal models of inflammatory disorders, including those of the nervous system are commonly used to explore the pathophysiological role of immune cell response in disease triggering and course and to develop biotechnology products for therapeutic use. Modeling some of these disorders, particularly neurodegenerative diseases, implies surgical manipulations for the intracerebral introduction of disease-initiating substances (toxins, amyloids etc.). Design of these experiments involves the use of sham-operated animals as a control of non-specific intrinsic side-effects elicited by surgical manipulations *per se*, including local and systemic inflammation, where phagocytic cells are key participants. Short-term post-surgical immunomodulatory effects are widely reported. However, no study thus far has examined the long term effects of sham-surgery on phagocyte functions.

The *purpose* of this study was to evaluate the effect of sham-surgery, commonly used for modeling neurodegenerative diseases, on phagocyte functions in the far terms after the surgical manipulations.

Materials and Methods. Adult male Wistar rats were used in the study. Sham surgery consisted of stereotactic unilateral injection of saline solution into the median forebrain bundle (sham-operated 1, SO1) or directly into the substantia nigra (sham-operated 2, SO2). Before the placebo surgery, animals were anaesthetized using nembutal and ketamine/xylazine correspondingly. Functional characteristics (phagocytic activity, oxidative metabolism, CD80/86 and CD206 expression) of phagocytes (microglia, peritoneal macrophages, circulating monocytes and granulocytes) were examined by flow cytometry. Differential leukocyte count was conducted using hematological analyzer.

Results. Phagocytes from animals underwent of different protocols of placebo surgery, demonstrated various patterns of functional changes on day 29 after the manipulations. In animals from SO1 group, we observed signs of residual neuroinflammation (pro-inflammatory shift of microglia functional profile) along with ongoing resolution of systemic inflammation (anti-inflammatory metabolic shift of circulating phagocytes and peritoneal macrophages). In rats from SO2 group, pro-inflammatory polarized activation of peritoneal phagocytes was registered along with anti-inflammatory shift in microglia and circulating phagocytes.

Conclusions. Sham surgery influences functions of phagocytic cells of different locations even in the far terms after the manipulations. These effects can be considered as combined long-term consequences of surgical brain injury and the use of anesthetics. Our observations evidences that sham associated non-specific immunomodulatory effects should always be taken into consideration in animal models of inflammatory central nervous system diseases.

Key words: sham surgery; phagocytes; neuroinflammation; systemic inflammation.

Animal experiment in biomedical research is a subject of debates in view of the growing awareness of ethical questions, and as a result of a gradually changing place of animals in our society. Nevertheless, the use of animal models currently stays a mandatory

practice in medicine and veterinary, as well as in pharmaceutical biotechnology [1–2]. In particular, animal models of inflammatory disorders of the nervous system are commonly used to explore the pathophysiological role of immune cell response in disease triggering and course and to develop biotechnology products for therapeutic use. The regulations concerning the use of animals for scientific purposes require making it under restrictive conditions, taking into account 3Rs — replacement, reduction, and refinement — expressed in 1959 by Russel and Burch [3]. One of the aspects of controversy regarding these regulations is the use of sham surgery (placebo surgery) in animal models of diseases. Sham surgery is a false surgical intervention that leaves out the step thought to be therapeutically or experimentally necessary. Historically, researchers have used sham-operated animals in numerous models of human diseases (cardiovascular, orthopedics, central nervous system diseases etc.). In particular, modeling of neurodegenerative diseases including Parkinson's (PD) and Alzheimer's diseases implies surgical manipulations for the intracerebral introduction of disease-initiating substances (toxins, amyloids etc.) [4, 5]. Ethical reasons demand consideration of unmanipulated (unoperated, intact) control animals to minimize animal suffering and unnecessary procedures [6]. However, surgical manipulation *per se* triggers intrinsic side effects, including local tissue damage, inflammation, and wound healing. In addition, non-physical factors can also influence inflammatory response to surgical stress [7, 8]. Therefore, sham surgery control groups are essential to eliminate the influence of these effects on the study results evaluation [9].

Phagocytes — circulating neutrophils and monocytes, as well as tissue-resident macrophages — are key participants of the onset, progression and resolution of inflammation involved inter alia in tissue damage and wound healing [10]. Resident macrophages drive local tissue inflammation and are to a large extent responsible for recruiting of circulating phagocytes into the inflamed area [11, 12]. Literature data and our own results amply evidence, that metabolic features of circulating phagocytes mirrors the course of systemic inflammatory response [13–15]. According to current concept concerning phagocyte plasticity, these cells respond to different environmental stimuli by polarized activation. In this process, phagocytes can acquire two polar functional states: M1 (pro-inflammatory) and M2 (anti-inflammatory), as well as numerous intermediate functional states. M1 polarized activation is associated with increased microbicidal capacity and enhanced secretion of pro-inflammatory cytokines to ulterior initiation and enhancement the cell-mediated adaptive immunity. M2 polarized activation endows phagocytes with ability to participate in antiparasitic immune responses, as well as in allergic reactions, wound healing, resolution of inflammation, and tissue remodeling [16, 17].

Surgical manipulations elicit local and systemic response to injury that leads to alterations in the immune and circulatory systems, including phagocyte function changing. These alterations can manifest in the form of temporary postoperative immunosuppression or post-surgery inflammatory response [18, 19]. All reports concerning postoperative immunomodulation describe fluctuations of immune system cells including phagocytes over short-term period after the surgical manipulations including sham-surgery. However, no study thus far has explored the potential long-term impact of sham surgical procedures on phagocyte functions. The purpose of this study was to evaluate the effect of sham-surgery, commonly used for modeling neurodegenerative diseases, on phagocyte functions in the far terms after the surgical manipulations.

Materials and Methods

Animals and study design. The study was conducted on adult male Wistar rats (220–250 g) bred in the vivarium of the Educational and Scientific Centre "Institute of Biology" of Taras Shevchenko National University of Kyiv, Ukraine. The animals were kept in standard conditions with *ad libitum* access to water and standard diet. Animal protocol was approved by the University Ethics Committee according to Animal Welfare Act guidelines. All procedures with animals were performed in conformity with the principles of humanity as it was written in "General principles of animal experimentation" approved by the National Congress on bioethics (Kyiv, 2001–2007) and in conformity with Council directive of November 24, 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (86/609/EEC). Forty rats were used

in this study. Before the experiment, animals were randomized by weight and divided into three groups: I- intact (n = 20), II — sham-operated 1 (n = 10), and sham-operated 2 (n = 10). At the day of surgery, rats from groups II were anesthetized with nembutal (50 mg/kg, i.p., "Sigma", USA), placed in a stereotaxic instrument (SEJ-4, Ukraine), and injected unilaterally with 4 µl of 0.1% ascorbic acid in isotonic saline solution into the left lateral ascending bundle (2.2 mm caudal and 1.5 mm lateral from the bregma in accordance with the atlas coordinates). This protocol of sham surgery is commonly used in 6-OHDA-induced PD model. Namely, ascorbic acid is usually used as a stabilizer in order to prevent 6-OHDA oxidation [20]. Rats from groups III were anesthetized with a mixture of ketamine (75 mg/kg diluted in sterile water for injection, Sigma, USA) and 2% Xylazine (400 µl/kg, Alfasan International BV, Netherlands) i.p., and injected unilaterally (left side) with 10 µg of 0.9% sodium chloride in the volume of 2 µl into the substantia nigra (AP = −5.3; ML = ± 2.0; DV = −7.2) according to Hoban et al., 2013. This protocol of sham surgery is commonly used in bacterial lipopolysacharide-induced PD model. [21]. Solution was also injected into the brain tissue at a rate of 1 µl/min (every 15s). The injector was left in place for 5 min before slowly withdrawing it to allow for liquid diffusion and prevent reflux.

Hematological parameters, as well as phagocyte metabolic and functional characteristics were investigated on day 29 after the surgery, considering spontaneous regeneration of nigrostriatal dopaminergic neurons in animals 24–28 days after the toxin introduction [22]. Rats were sacrificed by cervical dislocation as a mode minimizing cell death in brain structures [23].

Hemogram analysis. Hematological indicators were determined using an analyzer "Particle counter model PCE 210" (ERMA, Japan), adapted for the study of blood cells in rats and mice. In addition to differential leukocyte count, systemic inflammation markers were calculated. Neutrophil to lymphocyte ratio (NLR) was defined as the absolute neutrophil count/ absolute lymphocyte count. Derived NLR (dNLR) was calculated as absolute neutrophil count/white blood cells total count. Lymphocyte to monocyte ratio (LMR) was defined as the absolute lymphocyte count/ absolute monocyte count. Systemic immune-inflammation index (SII) was defined as platelet count × NLR [24].

Microglia cell isolation

Microglia cells were isolated from whole brain tissue homogenates as described previously [25]. Brain was rapidly extracted on ice. Hippocampus was dissected and perfused using a phosphate buffered saline. Isolated tissue was softly dissociated in ice cold phosphate buffered saline (PBS) supplemented with 0.2% glucose using Potter homogenizer. Tissue homogenate was filtered through a 40 nm cell strainer (BD Biosciences Discovery) and sedimented at room temperature. After this, microglia cells were isolated in Percoll density gradient. Purity of isolated microglia fraction was evaluated by flow cytometry using FACSCalibur flow cytometer and CellQuest Pro software (Beckton Dickinson, USA), and using fluorescein isothiocyanate (FITC) mouse anti-rat CD11b (BD PharmingenTM) and phycoerythrin (PE) mouse anti-rat CD45 (BD PharmingenTM). The proportion of CD11b+CD45+ cells was 88.9 ± 3.7%. Cell viability was determined by Trypan blue exclusion test. The proportion of viable cells was ≥ 93%.

Peritoneal macrophage isolation

Peritoneal macrophages (PM) were isolated without preliminary sensitization as described previously [26]. PM were harvested using PBS containing 100 U/mL of heparin. Cells were sedimented at 300 g for 5 min at 4 °C, washed twice with serum–free DMEM, and resuspended in DMEM containing 10% fetal calf serum and 40 µg/mL gentamycin.

Phagocyte metabolic and phenotypic characteristics assessment

Phagocytic activity was detected as described earlier [14]. FITC-labeled thermally inactivated cells of *Staphylococcus aureus* Cowan I were used as an phagocytosis object. Blood/microglia/PM samples were incubated at 37 °C for 30 min with bacterial cells. Phagocytosis was arrested by adding a 'stop' solution (phosphate buffered saline with 0.02% EDTA and 0.04% paraformaldehyde). Data are presented as the percentage of phagocytizing cells (PP) and phagocytosis index (PhI) (the average fluorescence per phagocytic cell). The oxidative metabolism (reactive oxygen species (ROS) generation) of phagocytes was explored using 2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen) as described previously [14].

For phagocyte phenotyping, FITC-labeled anti-CD80/86, and phycoerythrin (PE)-labeled anti-CD206 antibodies (Becton Dickinson, Pharmingen, USA) were used. Results were assessed using FACSCalibur flow cytometer

and CellQuest software (Becton Dickinson, USA). Granulocytes and monocytes were gated according to forward and side scatter.

Statistical analysis

All data are presented as mean ± SD. Statistical differences were calculated using ANOVA with Tukey's post-hoc test. Differences were considered significant at $P \leq 0.05$.

Results and Discussion

Sham-surgery as a control for intracerebral drug introduction was associated with alterations in functional state of phagocytes of different locations in the far terms after the manipulations. Probably, one can regards these alterations as combined long-term consequences of surgical brain injury and the use of anesthetics. Encephalon belongs to immunologically privileged sites, and is isolated from the immune system by the blood-brain barrier (BBB). Instead, brain is equipped with autonomous immune system represented by 80% by microglial cells — resident brain phagocytes [27]. These cells are responsible for immune patrolling and immune surveillance in the brain tissue. In addition, these cells are key drivers of the neuroinflammation. However, recently it has become obviously, that immune-privileged nature of the brain is not absolute, and there is afferent transportation of brain antigens to the peripheral lymphoid tissues, which normally induces immune tolerance to these antigens. BBB weakening is accompanied by the increased exposure peripheral immune cells with brain antigens, and can be associated with the development of local and systemic inflammatory and autoimmune responses [28, 29].

To assess whether sham surgery initiated long-standing inflammatory response, we compared functional characteristics of microglia population in intact and lesioned animals. In our experiments, microglia from rats in different sham-groups demonstrated distinct patterns of functional alterations. PP in microglia population was increased in both sham-groups as compared with intact animals (Fig. 1, *A*), indicating activated state of these cells [30]. At the same time, phagocytic activity in sham-operated group 1 was lower than that in other two groups (Fig. 1, *B*). Microglia from these animals was also

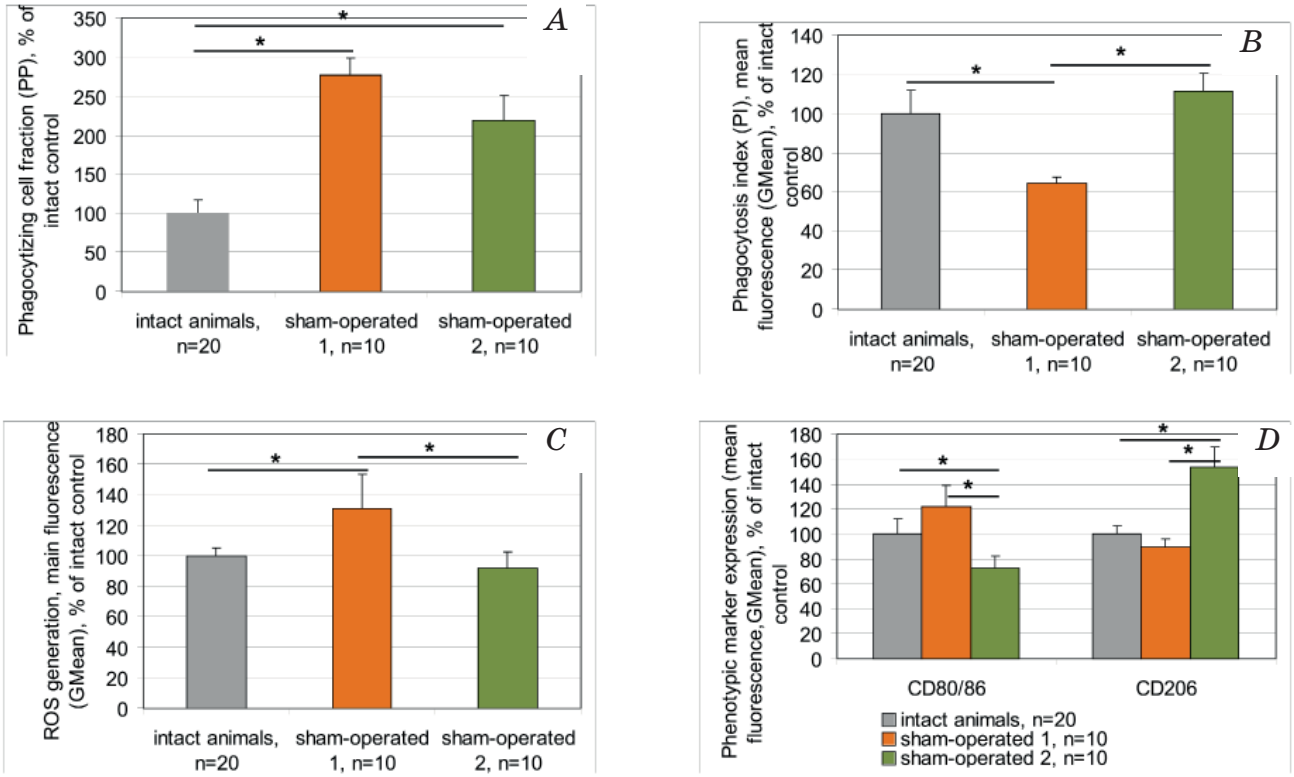

Fig. 1. **Functional and phenotypic characteristics of microglial cells of rats in the far terms after the sham-surgical manipulations:**

A, B — phagocytic activity; *C* — oxidative metabolism; *D* — phenotypic marker expression. Data are presented as Mean ± SD. * indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test)

characterized by increased ROS generation. Taken together, such results indicate residual pro-inflammatory shift of these cells [31]. One of the reasons of this effect can be the ability of sodium pentobarbital to induce local and systemic inflammation after the i.p. injection [32]. In microglial cells from rats in sham-operated group 2, down-regulated CD80/86 expression along with overexpression of CD206 was registered. It indicates moderate anti-inflammatory functional skew of these cells, probably, associated with the resolution of neuroinflammation caused by surgical brain injury.

Both the relative and absolute counts of circulating leukocytes, including myeloid cells, are key indexes for quantifying the magnitude of a systemic inflammation [22]. In our experiments, monocytosis was revealed in rats from sham-operated group 2 (Fig. 2, *A* and *B*). Our observations are in agreement with data reported by Hoffman et al. about that nonspecific inflammatory response to sham surgery could substantially effect on the pattern of systemic monocyte kinetics [33]. Monocytosis entailed the decrease of LMR value in rats from sham-operated group 2 (Table 1). Values of remaining indicators in sham-operated animals didn't differ significantly from those in intact rats.

Minor changes in quantitative indices of circulating phagocytes in sham-operated animals were associated with rather substantial alterations of their functions. Minor changes in quantitative indices of circulating phagocytes in sham-operated animals were associated with rather substantial alterations of their functions. In expanded monocyte fraction in rats from sham-operated group 2, increased phagocytizing cell fraction was detected (Fig. 3, *A*) with low phagocytic intensity (Fig. 3, *B*). It can indicate the recruitment of bone marrow derived monocytes, which might be released into the peripheral blood under stress conditions, and are characterized by lowered phagocytic activity [34]. ROS generation of circulating phagocytes in sham-operated animals from both groups was decreased as compared to intact animals (Fig. 3, *C*), whereas both CD80/86 and CD206 were overexpressed (Fig. 3, *D*). CD206 up-regulation is a phenotypic marker of phagocyte anti-inflammatory metabolic shift, whereas CD80/86 overexpression is a marker of pro-inflammatory metabolic shift [35]. Nevertheless, it is necessary to note that CD80 is also up-regulated in highly phagocytizing, CD206-overexpressing circulating myeloid-derived suppressor cells (MDSC) [36].

We inclined to suggest, that taken together metabolic and phenotypic characteristics of circulating phagocytes in rats in the far terms after the sham-surgical manipulations indicate ongoing resolution of systemic inflammation.

Peritoneal macrophages (PM) belong to the omentum-associated lymphoid tissue (Omentum-Associated Lymphoid Tissue, OALT), which has many features in common with Mucosa Associated Lymphoid Tissue (MALT) and is actively involved in initiating and controlling local and systemic inflammatory processes. OALT is closely related to the systemic vascular network and interacts with the central nervous system and the hypothalamic-pituitary-adrenal axis [37]. The biological characteristics of this tissue are not yet fully understood. However, there is ample evidence of the integrative role of PM and other OALT cells in the pathophysiology

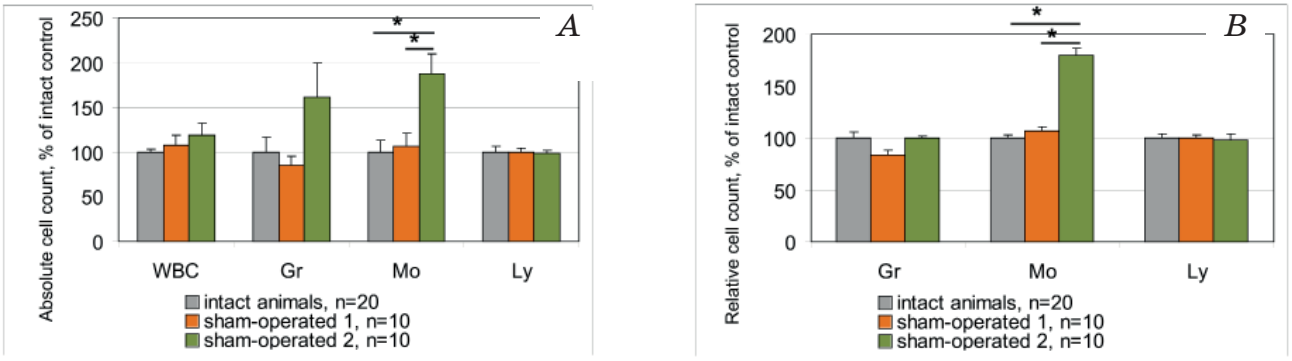

Fig. 2. **Differential leukocyte count in rats in the far terms after the sham-surgical manipulations. Data are presented as Mean ± SD. * indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test)**

Table 1. **Leukocyte ratios in rats in the far terms after the sham-surgical manipulations**

Animal group	Coefficients			
	NLR	dNLR	LMR	SII
Intact ($n = 20$)	0.32 ± 0.03	0.32 ± 0.07	11.0 ± 2.44	33.73 ± 9.09
Sham-operated 1 ($n = 10$)	0.25 ± 0.05	0.34 ± 0.12	8.45 ± 2.28	32.85 ± 8.04
Sham-operated 2 ($n = 10$)	0.25 ± 0.06	0.25 ± 0.07	5.52 ± 2.28*	41.91 ± 7.83

Note: * indicates significant ($P \leq 0.05$) differences in comparison with intact animals (ANOVA with Tukey post-hoc test), Mean ± SD. NLR — neutrophil to lymphocyte ratio, dNLR — derived neutrophil to lymphocyte ratio, LMR — lymphocyte to monocyte ratio, SII — systemic immune inflammation index.

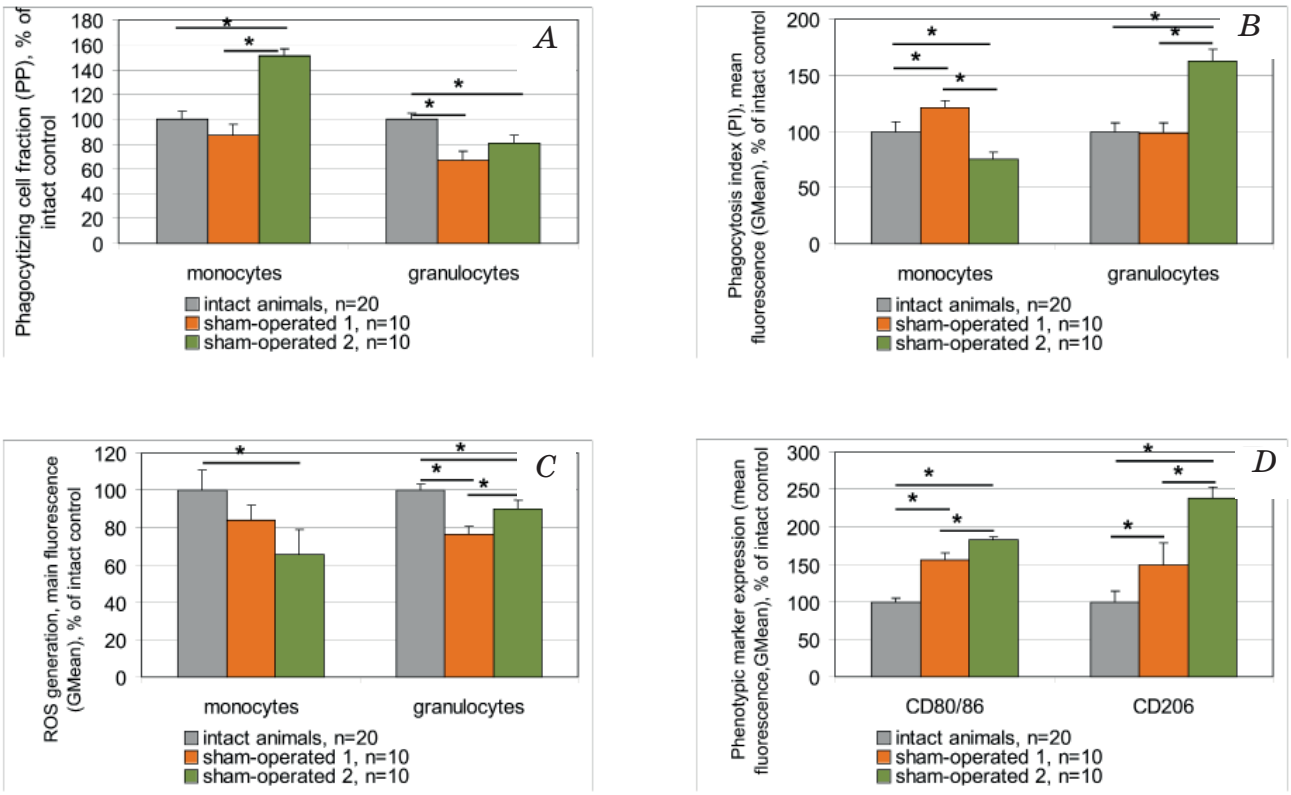

***Fig. 3*. Functional and phenotypic characteristics of circulating phagocytes of rats in the far terms after the sham-surgical manipulations:**

A, B — phagocytic activity; *C* — oxidative metabolism; *D* — phenotypic marker expression. Data are presented as Mean ± SD.

* indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test).

of inflammatory diseases. This circumstance as well as the fact that anesthetics in our experiments were introduced i.p. raised our interest to examining these cells in sham-operated animals. Metabolic and phenotypic characteristics of PM in sham-operated rats differed from those in intact animals in the far terms after the sham-surgical manipulations, and varied slightly between placebo-surgery groups. It might be stipulated by the difference in the effects of ketamine/xylazine and barbiturates on phagocytic cells, reported by Hristovska et al. [38]. In rats from sham-operated group 2, which received i.p.

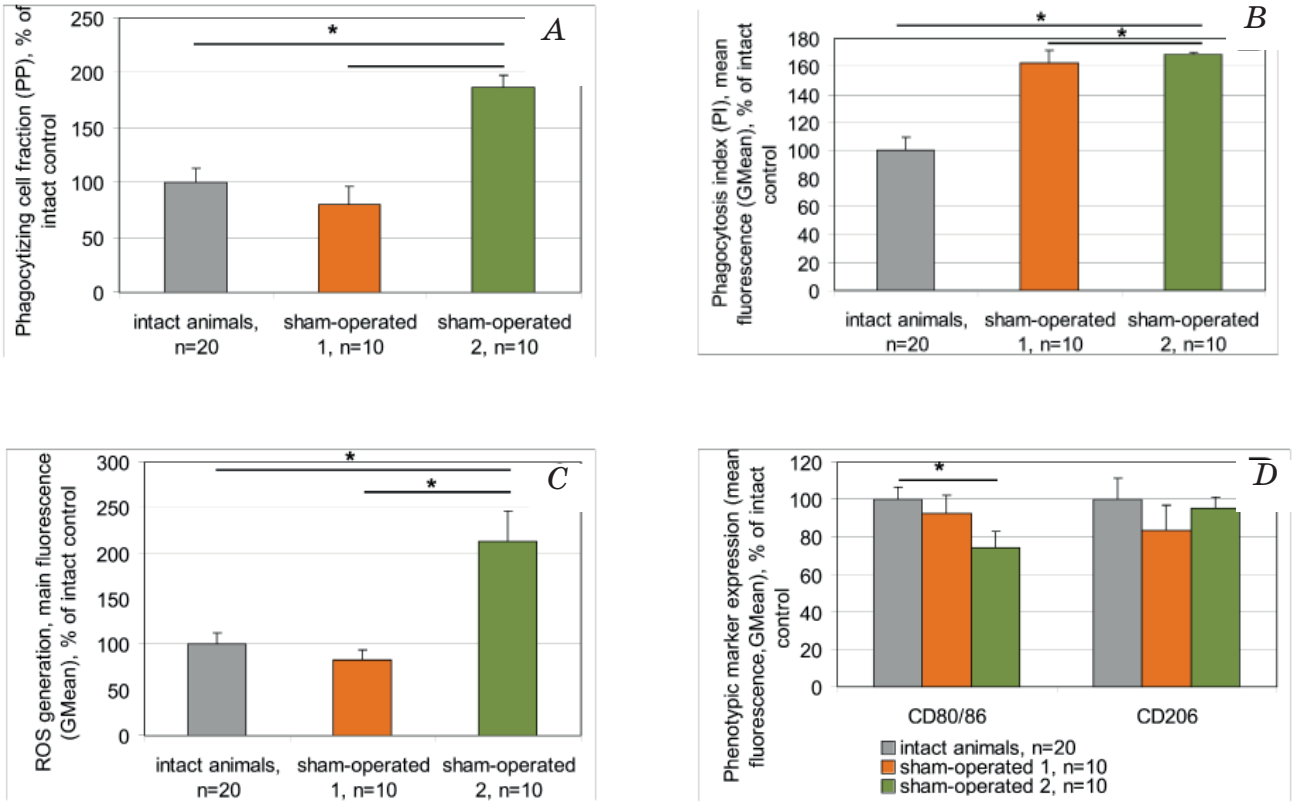

Fig. 4. **Functional and phenotypic characteristics of peritoneal macrophages of rats in the far terms after the sham-surgical manipulations:**
A, B — phagocytic activity; *C* — oxidative metabolism; *D* — phenotypic marker expression. Data are presented as Mean ± SD. * indicates significant ($P \leq 0.05$) differences (ANOVA with Tukey post-hoc test)

injection of ketamine/xylazine, we observed highly increased proportion of phagocytizing PM (Fig. 4, *A*).

These cells were characterized by up-regulated phagocytic intensity (Fig. 4, *B*) and augmented ROS generation (Fig. 4, *C*). CD80/86 membrane expression was decreased in these cells, the level of CD206 membrane expression didn't differ significantly as compared to intact animals. Although CD80/86 up-regulation indicates pro-inflammatory phagocyte metabolic shift, whereas down-regulation is a marker of anti-inflammatory polarized activation of phagocyte, overexpression of these co-stimulatory molecules is associated with acquiring antigen-presenting capacity by activated phagocytes, which is characteristic for terminal phases of inflammation. Phagocytes during earlier phases of inflammation are featured by decreased co-stimulatory molecules expression along with increased phagocytic activity and high production of reactive oxygen and nitrogen species [39]. Thus, metabolic and phenotypic characteristics of PM in rats from sham-operated group 2 evidence ongoing inflammation in peritoneal cavity 28 days after the sham-surgery with the use of i.p. ketamine/xylazine as anesthetics. Surprisingly, PM in rats from sham-operated group 1, which received i.p. injection of nembutal during placebo-surgery, demonstrated no substantial alterations in their functional and phenotypic characteristics excluding increased phagocytic activity, which can be considered as a sign of residual resolution of inflammation in peritoneal cavity.

In conclusion, our results exposed a hitherto underappreciated impact of sham-surgery on phagocytes of different subsets and location even in the far terms after the sham-surgical manipulations. Based on our results, we conclude that sham surgery associated confounding effects should always be taken into consideration when examining phagocyte subsets in animal models of inflammatory central nervous system diseases. Therefore, sham surgery control groups are mandatory for this purpose.

The study was supported by a project funded by the Ministry of Education and Science of Ukraine (State registration No. 0120U102130).

Acknowledgement

We would like to thank bachelor and master students of ESC "Institute of Biology and medicine" participated in this study, as well as Dr.Vitalina Svyatetskaya for the assistance in surgical manipulations.

Conflicts of Interest

Authors declare no conflict of interest.

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ДОВГОСТРОКОВІ ЕФЕКТИ ПЛАЦЕБО-ХІРУРГІЇ НА ФУНКЦІЇ ФАГОЦИТІВ У ЩУРІВ

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Експериментальне моделювання запальних захворювань, у тому числі нервової системи, зазвичай використовується для вивчення патофізіологічної ролі клітин імунної системи у виникненні та перебігу захворювання, а також для розробки біотехнологічних продуктів для терапевтичного застосування. Моделювання деяких запальних захворювань, зокрема нейродегенеративних, передбачає проведення хірургічних маніпуляцій із внутрішньомозкового введення тригерних субстанцій (токсинів, амілоїдів та ін.), що спричиняють розвиток захворювання. Схема цих експериментів включає використання хибнооперованих тварин, як контроль неспецифічних побічних ефектів, викликаних хірургічними маніпуляціями, як такими, у т.ч. місцевого та системного запалення, в якому ключовими учасниками є фагоцитарні клітини. Широко відомі короткострокові післяопераційні імуномодуляторні ефекти. Однак досі не вивчено довгострокових ефектів плацебо-хірургії на функції фагоцитів.

Метою дослідження було оцінити вплив плацебо-хірургії, що зазвичай використовується для моделювання нейродегенеративних захворювань, на функції фагоцитів у віддалені терміни після хірургічних маніпуляцій.

Матеріали та методи. У дослідженні використовували дорослих щурів-самців лінії Вістар. Плацебо-операція полягала в односторонньому стереотаксичному введенні фізіологічного розчину в серединний пучок переднього мозку (група 1) або безпосередньо в чорну субстанцію (група 2). Перед плацебо-операцією тварин анестезували нембуталом та кетаміном/ксилазином, відповідно. Функціональні характеристики (фагоцитарну активність, оксидативний метаболізм, експресію CD80/86 та CD206) фагоцитів (мікроглії, перитонеальних макрофагів, моноцитів та гранулоцитів, що циркулюють) досліджували методом проточної цитометрії. Диференціальний підрахунок лейкоцитів проводили за допомогою гематологічного аналізатора.

Результати. Фагоцити тварин, що зазнали різних протоколів плацебо-операцій, демонстрували різний характер функціональних змін на 29 добу після маніпуляцій. У тварин із групи 1 ми спостерігали ознаки залишкового нейрозапалення (прозапальний зсув функціонального профілю мікроглії) поряд з триваючим завершенням системного запалення (протизапальний метаболічний зсув циркулювальних фагоцитів і перитонеальних макрофагів). У щурів з групи 2 реєстрували прозапальну поляризовану активацію перитонеальних фагоцитів поряд з протизапальним зсувом мікроглії та циркулювальних фагоцитів.

Висновки. Плацебо-хірургія впливає на функції фагоцитуючих клітин різної локалізації навіть у віддалені терміни після маніпуляцій. Ці ефекти можна розглядати як комбіновані віддалені наслідки хірургічної травми головного мозку та застосування анестетиків. Наші спостереження свідчать про те, що неспецифічні імуномодуляторні ефекти плацебо-хірургії завжди слід брати до уваги при експериментальному моделюванні запальних захворювань центральної нервової системи.

Ключові слова: плацебо хірургія; фагоцити; нейрозапалення; системне запалення.

UDC 557.112 + 577.121 https://doi.org/10.15407/biotech15.02.047

ADAPTOR PROTEIN RUK/CIN85 IS INVOLVED IN THE GLUCOSE METABOLISM REPROGRAMMING IN BREAST CANCER CELLS

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Received 19.04.2022
Revised 27.04.2022
Accepted 29.04.2022

To survive under hypoxia and lack of nutrients and to efficiently metastasize, cancer cells are able to modify their metabolism. Moreover, cancer stem cells (CSCs) were reported to have elevated glycolysis and altered glutamine and fatty acids metabolism [1]. Our previous findings demonstrated that overexpression of adaptor protein Ruk/CIN85 in breast cancer cells led to increased motility, invasiveness, and the manifestation of the CSCs features [2, 3].

Aim. This study aimed to investigate the changes in glucose metabolism in mouse 4T1 breast adenocarcinoma cells with different levels of Ruk/CIN85 expression.

Methods. We used 4T1 cells with stable overexpression (subline RukUp) or knockdown (subline RukDown) of Ruk/CIN85, as well as corresponding vector control sublines Mock and Scr. Cells were cultured in the complete RPMI-1640 medium under standard conditions. mRNA expression levels were estimated by RT^2-PCR, enzymes activities were measured by spectrophotometric and/or fluorometric assays.

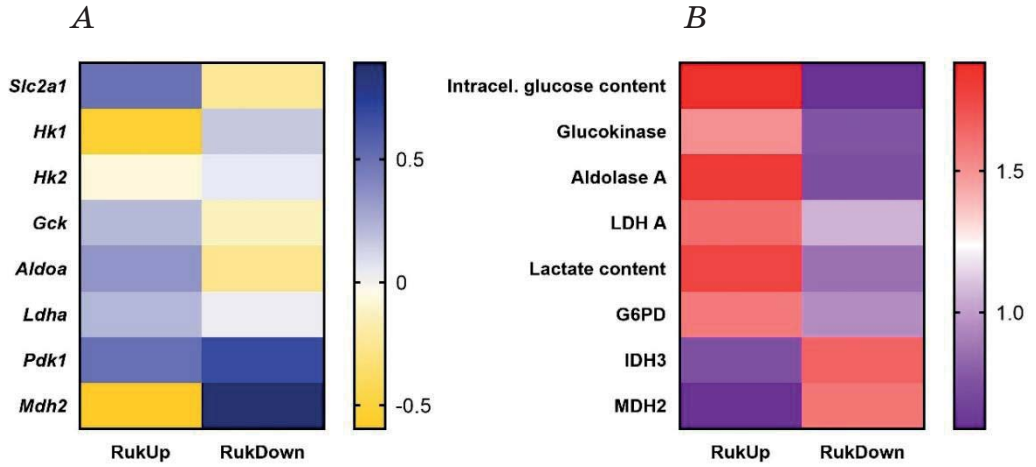

Fig. **Heatmap representation of gene expression (*A*) and enzyme activities or metabolites content (*B*) in 4T1 cells with overexpression and downregulation of the adaptor protein Ruk/CIN85 (*GraphPad Prism*)**

Results. Analysis of mRNA expression of glucose metabolism-related genes in RukUp and RukDown cells revealed that glycolysis genes are preferentially overexpressed in RukUp cells, and downregulated in RukDown cells (Fig. *A*). Thus, RukUp cells were characterized by significantly overexpressed *Slc2a1, Gck, Aldoa,* and *Ldha,* while in RukDown cells these genes were either down regulated or not changed. However, the expression of TCA (tricarboxylic acid) cycle enzyme *Mdh2* increased dramatically (by 7,8 times) in RukDown cells. These findings were confirmed and complemented by enzyme activities and metabolites analysis. Fig. *B* clearly indicates that high level of Ruk/CIN85 is strongly associated with elevated glycolysis, and low level of Ruk/CIN85 — with TCA and mitochondrial oxidation. In detail, we observed statistically significant changes in the activity of all studied enzymes in RukUp cells (increase by 1.5–1.9 times for glycolysis enzymes and G6PD, and decrease by 1.33–1.69 times for TCA enzymes). However, in RukDown cells we did not find any significant changes in glycolysis enzymes activities, but activities of mitochondrial IDH3 and MDH2 were elevated by 1.65 and 1.59 times, respectively.

Discussion. In this study we found that high expression level of Ruk/CIN85 is strongly associated with elevated glucose uptake, increased glycolysis, and diminished mitochondrial functioning. These features are characteristic of various highly aggressive malignant tumors and are known as the Warburg effect. Elevated glucose oxidation via glycolysis provides plenty advantages for cancer cells, such as fast ATP production, essential metabolic intermediates biosynthesis, avoiding oxidative stress, etc, that allows us to consider glycolysis is a promising target for anti-cancer therapy [4]. In addition, Ruk/CIN85 knockdown resulted in decreased glucose uptake and its elevated oxidation in the TCA cycle which is characteristic for normal epithelial cells.

Conclusions. The results obtained indicate that adaptor protein Ruk/CIN85 is involved in the metabolic reprogramming during breast cancer progression. High level of Ruk/CIN85 expression is associated with potentiation of the Warburg effect.

Key words: breast cancer, the Warburg effect, adaptor proteins, Ruk/CIN85, metabolic reprogramming.

Funding source. This research was partially supported by the NRFU grant 2020.02/0195 “Differential control of regulatory networks involved in the maintaining of epithelial-mesenchymal plasticity of breast adenocarcinoma cells”.

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UDC 577.152.1:[576/311/347:612.46]:613.24

https://doi.org/10.15407/biotech15.02.049

ACTIVITY OF AMP DEAMINASE AND 5′-NUCLEOTIDASE IN THE CYTOSOLIC KIDNEY FRACTION OF RATS UNDER THE CONDITIONS OF DIFFERENT PROTEIN AND SUCROSE CONTENT IN A DIET

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Receivd 18.04.2022
Revised 22.04.2022
Accepted 29.04.2022

It is shown that metabolic disorders under the conditions of excessive consumption of sucrose occur in the cells of the liver, kidneys, accompanied by the development of hyperglycemia [1]. Persistent chronic hyperglycemia leads to the development and progression of diabetic nephropathy. Homeostatic function of the kidneys is provided by a number of processes that require a significant expenditure of ATP energy — maintenance the balance of electrolytes and acid-base status, excretion of toxic substances, reabsorption of nutrients [2]. The functional activity of the kidneys is largely determined by the state of the energy supply system. Enzymes of the purine nucleotide cycle, 5′-nucleotidase (EC 3.1.3.5) and AMP deaminase (EC 3.5.4.6) enable metabolic transformations of purine nucleotides and control the levels of specific intracellular modulators: AMP, adenosine, and inosine.

Aim. The goal of this work was to evaluate the activity of AMP deaminase and 5′-nucleotidase in the cytosolic kidney fraction of rats under the conditions of different protein and sucrose content in a diet.

Methods. In the study, 10–12 week old white nonlinear rats weighing 130–140 g were used. The animals were separated into solitary plastic cages and *ad libitum* access to water. The animals were divided into the following experimental groups (n = 9): C –control; LP — animals receiving low-protein ration; HS — animals receiving high-sucrose diet; LP/HS — animals receiving low-protein high-sucrose diet. The activity of 5′-nucleotidase was evaluated based on the amount of inorganic phosphorus released in AMP hydrolysis and expressed in P*i* nmol per 1 min per 1 mg protein. AMP deaminase activity was determined using spectrophotometry by monitoring the increase in optical density at λ = 265 nm every 10 s for 1 min.

Results. The studies showed that in the cytosolic fraction of the kidneys of animals maintained on a low-protein diet, AMP-deaminase activity did not change significantly compared to the control (Fig. *A*). At the same time, in rats kept on a high-sucrose diet an almost 4-fold increase in AMP-deaminase activity compared with the control was found. However, in rats kept on a low-protein/high-sucrose diet AMP-deaminase activity in the kidney exceeded control values but was lower than in animals maintained on a high-sucrose diet. At the same time, we found that in rats kept on a high-sucrose diet the 5′-nucleotidase activity increased by about 2 times compared with the control, while in animals maintained on a low-protein/high-sucrose diet it reached its maximum exceeding the control by more than 2.7 times (Fig. *B*).

Discussion. The established activation of AMP deaminase and 5′-nucleotidase as a result of the excessive consumption of sucrose is likely to be accompanied by an increase in AMP degradation and can be considered as a mechanism of inhibition of AMP-activated protein kinase (AMPK) [3]. AMPK acts as a metabolic “switch” for energy metabolism at the cellular level. In several studies, AMPK activators attenuate diabetic nephropathy and improve high fat-induced kidney disease in mice. At the same time, a switch from AMP degradation to the formation of adenosine with the participation

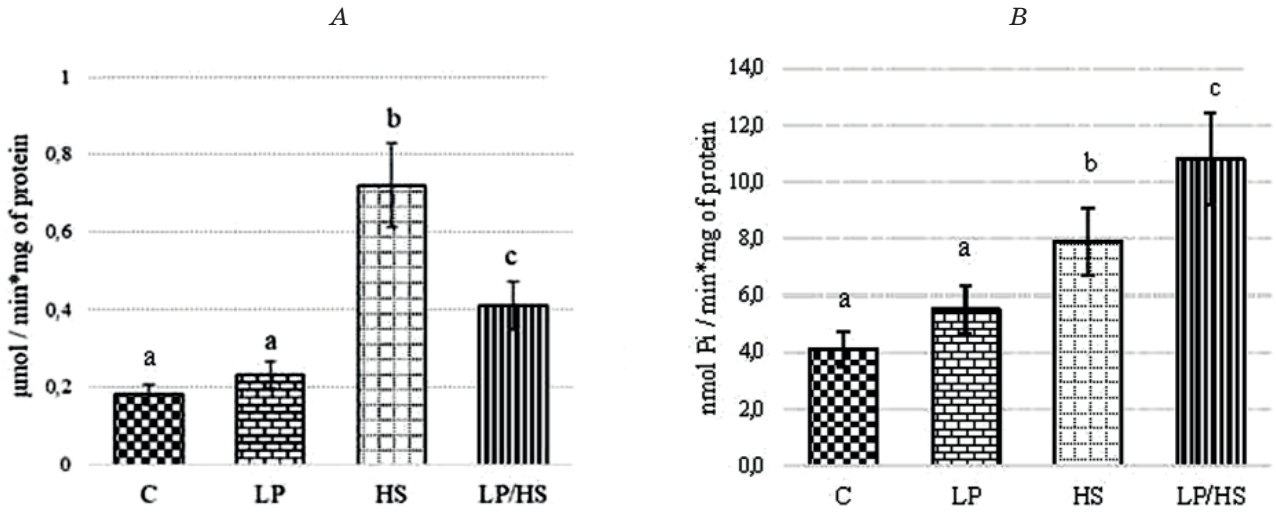

Fig. **Activity of AMP deaminase (*A*) and 5′-nucleotidase (*B*) in the cytosolic kidney fraction of rats under the conditions of different protein and sucrose content in a diet**

Different letters indicate significant differences inside the parameters and the same letters indicate no difference at $P \leq 0.05$.

of 5′-nucleotidase was found in the cytosolic fraction of the kidneys of animals kept a low-protein/high-sucrose diet. It probably has an important regulatory effect, since adenosine is considered an extracellular signaling molecule involved in many biochemical processes of the maintenance and restoration of the tissue homeostasis. Under the conditions of hyperglycemia, the formation of adenosine as a signalling molecule participates in the maintenance of filtration pressure, regulation of the reabsorption of water and nutrients, and inhibition of inflammation and fibrosis in the kidneys. Moreover, the increased activation of 5′-nucleotidase upon consumption of a low-protein/high-sugar diet may result in the intensified formation of inosine — a regulator of the expression of a number of proteins, which indicates an important role of this molecule in antioxidant defence activation and transmission of intracellular signals.

Conclusions. The excessive consumption of sucrose against the background of alimentary protein deficiency is accompanied by an increase in AMP-deaminase and 5′-nucleotidase activity in the cytosolic fraction of rat kidneys, which can be considered as a compensatory mechanism aimed at switching metabolic transformations in conditions of nutritional imbalance.

Key words: AMP desaminase, 5′-nucleotidase, kidney, low-protein diet, high-sucrose diet.

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UDC 57.088.2 **https://doi.org/10.15407/biotech15.02.051**

HPLC DETECTION OF ANTITHROMBITIC CALIX[4]ARENE IN BLOOD PLASMA OF ANIMALS

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Received 22.03.2022
Revised 15.04.2022
Accepted 29.04.2022

Previously sodium salt of 5,11,17,23-bis (dihydroxyphosphoryl) methylcalix[4]arene (C-145) was shown to be promising antithrombotic agent and was successfully tested *in vivo* [1, 2].

Aim. This work was focused on the development of the method for the direct detection of this substance in blood plasma and estimation of pharmacokinetics of this compound.

Methods. Wistar rats and outbreed rabbits were kept in the vivarium of Bila Tserkva National Agrarian University on a standard diet. C-145 was injected into the rat's lateral tail vein and into rabbit's marginal vein of the ear (12 mg/kg) or was administrated per-oral. The anticoagulant effects of C-145 in blood plasma were confirmed by activated partial thromboplastin time (APTT) test. HPLC was performed using Agilent 1100 series (Agilent, USA) on the phase cyano ZorbaxCN Column which parameters were L×I.D. 25 cm×4.6 mm.

Results. The maximal antithrombotic effect after the intravenous or per-oral administration of C-145 was observed after 4–6 hours. In particular clotting time in APTT-test in these blood plasma samples was prolonged trice and more (120 s against 46 s in control). Normalization of blood clotting was achieved after 24 hours after the injection.

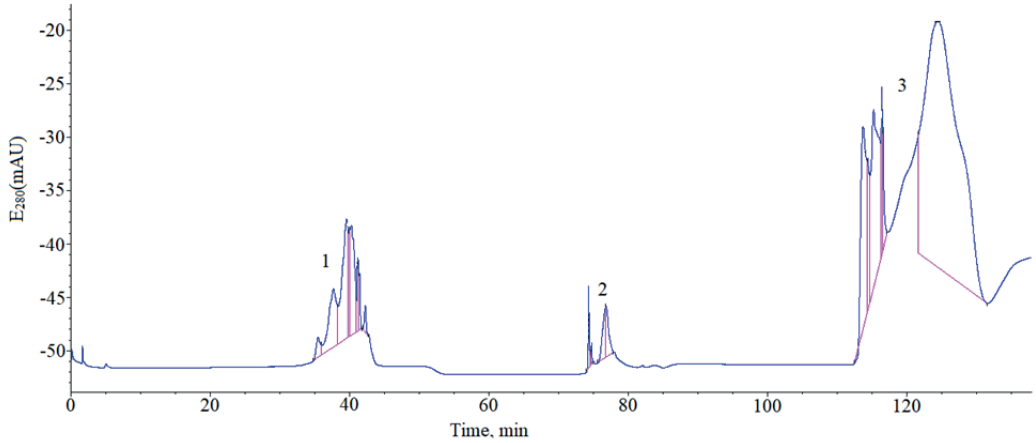

Fig. **HPLC chromatography of detection of calix[4]arene C-145 in rabbit blood plasma after 2 hours of intravenous injection with the use of combined linear gradient**
(acetonitrile 1 % → 100 % — from 30 till 40 minutes, and citrate buffer (0.1 M, pH 6.0) 0 % → 100 % — from 110 till 120 minutes using nitrile column Zorbax CN 25 cm, 4.6 mm, speed of the flow 1 ml/min, t = + 40 °C, UV detection at 280 nm. Fraction 3 — zone of calix[4]arene C-145 elution.

To develop a method for direct C-145 detection in blood plasma we selected samples with maximal prolongation of clotting time. For accurate analysis of blood plasma samples proteins were saturated by 10% trichloroacetic acid. After neutralization by $NaHCO_3$ samples were prepared using 12-port vacuum unit for solid-phase extraction (Agilent, USA) with a Bond-Elut C18 cartridge. The samples that contained C-145 were eluted by 100% methanol for the HPLC analysis performed on the phase cyano ZorbaxCN Column equilibrated with an acetonitrile solution (ddH_2O:AcCN 99:1). Elution was performed using a combined gradient of acetonitrile (100%) and citrate buffer (0.1 M, pH 6.0). The elution zone of C-145 was detected on the 128^{th} minute at 280 nm.

Discussion. C-145 was detected in blood plasma samples of animals after intravenous or per-oral administration of C-145 (Figure). Control blood plasma did not contain the peak of C-145, but it was detected clearly in the buffer solution of C-145 prepared *in vitro*. The presence of C-145 in blood plasma was correlated to the clotting time of the sample.

Conclusion. Application of the developed methods allowed us to confirm the direct antithrombotic effect of calix[4]arene C-145 on blood of experimental animals during intravenous administration. Also HPLC technique enabled to detect this substance in blood plasma and most likely could be applied for other biological solutions and could be modified for the quantitative analysis in the pharmacokinetic studies as well.

Key words: calix[4]arene, blood plasma, thrombosis, fibrinogen, HPLC.

Acknowledgement. Authors cordially acknowledge Prof. Rublenko for his brilliant supervision of animal studies.

Funding source. Study was financed by project No. 0121U110361 of National academy of sciences of Ukraine "Development of the prototype of antithrombotic preparation Calix[4]arene C-145 and its pre-clinical studies".

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UDC 617.711-002-022.7+577.112 https://doi.org/10.15407/biotech15.02.053

PROTEIN MARKERS OF HYPOXIA AND ANGIOGENESIS IN TEAR FLUID OF PATIENTS WITH TRAUMATIC CORNEAL INJURY

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Received 22.02.2022
Revised 16.04.2022
Accepted 29.04.2022

The cornea is an avascular, transparent tissue that is essential for visual function. Penetrating or perforating ocular injuries as well as infectious keratitis can cause corneal ulceration, scarring, and, in more severe cases, partial or complete vision loss. Since the vast majority of patients with traumatic corneal injuries are people of working age, this causes an important medical and social burden [1]. Numerous modulators coming from tears, inflammatory cells, extracellular matrix (ECM), neural cells, corneal epithelial cells, or stromal fibroblasts can regulate the complex wound healing process [2]. Biomarker evaluation in tear fluid may provide valuable insight into diagnosis of disease, progression or modulation of disease with and without pharmaceutical intervention, thus making ocular biomarker assessment critical component of ophthalmic drug discovery and development. It was currently reported that the tear proteome consists of about 1800–2000 proteins [3]. Among them, some specific proteins can be used as relevant markers of hypoxia and angiogenesis, which are often developed in the injured cornea and contribute to chronic corneal wound healing. Thus, the aim of our study was to evaluate tear levels of some protein endpoints that can reflect intensities of hypoxia, angiogenesis and tissue remodeling in wounded cornea.

Methods. We examined 21 patients (21 eyes) with nonpenetrating corneal injuries, which were observed in the clinic of "Alexander Clinical Hospital" that is a clinical base of the Bogomolets National Medical University in Kyiv for the period 2020–2021. The study was approved by the local ethical committee of Bogomolets National Medical University and the research is complied with Helsinki Declaration. Demographic and clinical characteristic of the patients are presented in the Table 1. Patients underwent standard ophthalmological examination including previous history and ocular symptoms, visual acuity test, complete anterior and posterior eye segments examination using slit lamp biomicroscopy, evaluation of corneal staining with fluorescein, ophthalmoscopy. Healthy volunteers ($n = 10$) served as a control.

Tear fluid was collected from patients and control volunteers with the use of a disposable tip micropipette. From the lower arch of the conjunctiva without instillation of anesthetic, tears were collected in a sterile plastic Eppendorf tube and frozen at −20 °C before laboratory examination. Proteins of tear fluids were separated by SDS-PAGE (loading 50 μg total protein per track). Then, levels of hypoxia inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), and angiostatins were measured by western blot. Active MMP-9 levels were evaluated by gelatin zymography. The results of blot and zymography assays were processed by densitometric software and then analyzed statistically with the use of Mann-Whitney *U*-test. Values are represented as the mean ± SD. $P < 0.05$ was regarded as significant for all statistical analyses.

Results. Elevated HIF-1α ($P < 0.001$) and angiostatins ($P < 0.05$) levels were revealed by western blot in tear fluid samples collected from patients with injured cornea in comparison with the control group (Fig. 1). It is noteworthy that extremely low amounts of VEGF were detected in tear fluid from injured eyes, in spite of abundance of its transcription inducer HIF-1α.

Dramatically increased levels of active MMP-9 were found in the tear fluids of patients with corneal wounds, while no significant collagenolytic activity was observed in tears from healthy eyes (Fig. 2). There is a strong correlation between extent of corneal lesions and changes in markers expression.

Table. **Demographic and clinical characteristic of patients with corneal injuries**

Characteristics	Values
Gender Male Female	 13 (61.9%) 8 (38.1%),
Age	43.5 ± 2.2
Visual acuity	From 0.08 to 0.9
Location of corneal damage Central (optic zone) Paracentral	 9 (42.9%) 12 (57.1%)
Depth of injury Superficial Deep	 17 (81%) 4 (19%)
Pericorneal injection Mild Severe	 11 (52.4%) 10 (47.6%)

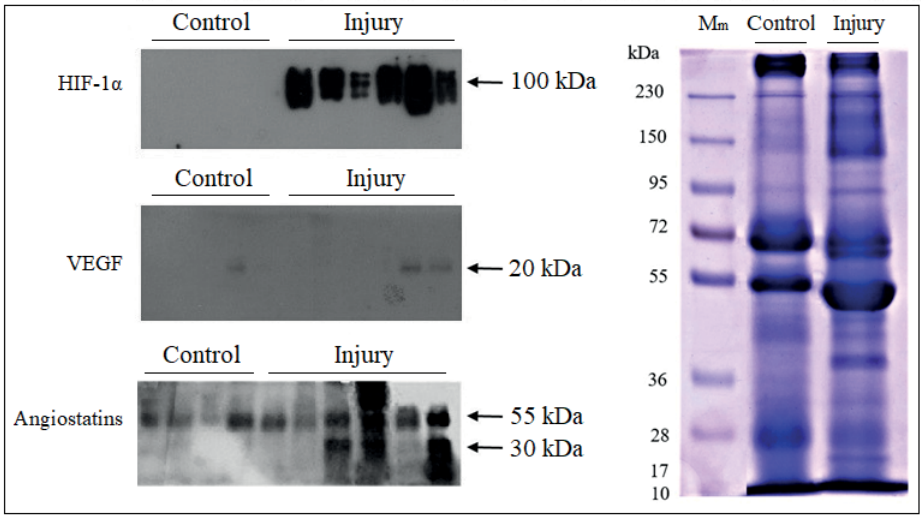

Fig. 1. **Representative blotograms of marker proteins in tear fluid of healthy volunteers and patients with ocular trauma**
right — Coomassie-stained SDS-PAGE of tear fluid proteins.

Discussion. Corneal function is vital for normal vision and includes barrier protection, light refraction, and ultraviolet light filtration. Because the cornea is the main refractive surface of the eye, even minor changes in its contour lead to significant vision problems. The corneal epithelium is maintained in a complex balance that can be easily disrupted. Hypoxia, which is developed in wounded cornea, affects the cornea in multiple aspects, including disturbance of the epithelium barrier function, corneal edema due to endothelial dysfunction and metabolism changes in the stroma, and thinning of corneal stroma. HIF-1α is the key protein facilitating hypoxia-induced changes is injured cornea [4]. VEGF is an angiogenic regulator that has been identified as an important pathophysiologic mediator in the development and maintenance of angiogenesis seen in neovascular eye disease, while angiostatins, plasminogen-derived fragments, are known to counteract VEGF-induced pro-angiogenic signaling and impede new vessel growth [5, 6]. Low VEGF levels in tear fluids from traumatic eyes can be explained by retention of VEGF in corneal tissue, where it governs pro-angiogenic signaling, or down-regulation of its expression by enhanced angiostatins [7]. Thus, corneal wound healing process is characterized by cellular remodeling and changes in protein tear composition in preparation for healing. This results in an increased production of proteolytic enzymes (including MMP-9), which degrade the damaged epithelial basement membrane. MMPs decrease cellular adhesion and help enhance cellular migration. In normal cornea, MMPs are responsible for the precise organization

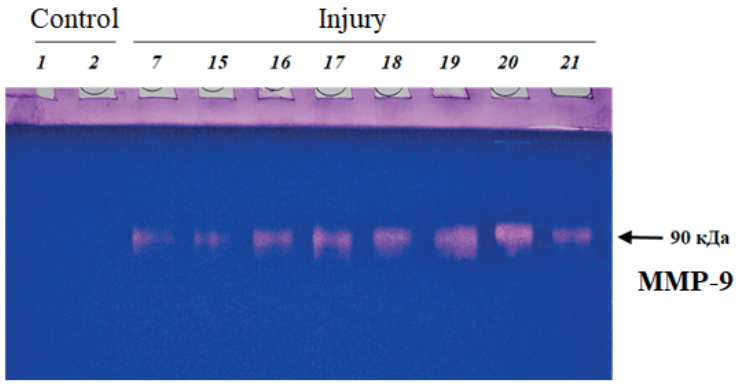

Fig. 2. **Representative gelatin zymography of matrix metalloproteinase -9 (MMP-9) in tear fluid of healthy volunteers and patients with corneal trauma**

of collagen fibrils within the corneal stroma, which is imperative to maintaining corneal clarity and appropriate stromal hydration. However, an excessive increase in MMPs activity may result in abnormal degradation of the extracellular matrix, inhibition of reparative angiogenesis through plasminogen degradation and angiostatin formation that hinder proper corneal wound healing [8]. Thus, our data demonstrate that tear levels of hypoxia/angiogenesis markers, along with MMP activity, can be helpful as ocular biomarkers to diagnose and assess corneal wound healing. In the future, the diagnostic power of these and other plausible markers in tear fluid should be verified in the different validation sets.

Conclusions. Tear levels of HIF-1α and angiostatin as well as MMP-9 activity could represent valuable biomarkers of corneal injury severity in traumatic eye.

Acknowledgement. Authors are grateful to Dr. A. A. Tykhomyrov and Dr. D. G. Zhaboedov for technical support and fruitful discussion.

The authors state that they have no conflict of interest.

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UDC 598.115.33 https://doi.org/10.15407/biotech15.02.056

ACTION OF VENOM OF VIPERA SNAKE OF UKRAINE ON BLOOD COAGULATION *in vitro*

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Received 02.03.2022
Revised 27.03.2022
Accepted 29.04.2022

Hemorrhagic action caused by phospholipases is the main toxic action of *Vipera* snakes venom [1]. However, action on the blood coagulation system is studied less broadly.

Aim. In this study we focused on the search of fibrinogen-targeted proteases in the venom of *Vipera renardi, Vipera nikolskii* and *Vipera berus*. Venom of *Vipera berus* was also fractionated on Q-sepharose and action of separated fractions on human blood plasma, platelets and red cells was studied.

Methods. Analysis of protein mixtures was performed using SDS-PAGE. Action on the blood coagulation system was analyzed using the APTT assay. Identification of protein components with fibrinolytic activity was performed using enzyme-electrophoresis with fibrinogen as the substrate [2]. Fractionation of *V. berus* venom was performed on Q-sepharose using FPLC system Acta Prime. Action of separated fractions on ADP-induced platelet aggregation in platelet rich blood plasma was analyzed using Aggregometer AP 2110 [3]. Hemolytic action of fractions was estimated using fresh human red cells. Amount of released hemoglobin was estimated by spectrophotometry on Optizen POP.

Results. All studied venoms had different protein compositions with major protein fractions in the range from 25 kDa to 130 kDa. Both *V. berus* and *V. nikolskii* venoms taken in 1:200 dilutions reduced the time of clotting in APTT test from 25 to 13 s. In contrast, *V. renardi* venom in the same dilution prolonged the clotting time from 25 s to 180 s that we assumed as the result of fibrinogen-specific protease presence. According to enzyme-electrophoresis data all studied venoms contained fibrinogen-specific proteases with the apparent molecular weights for *V. berus, V. nikolskii* — 25–55 kDa. and *V. renardi* — 55–75 kDa. Fractionation of crude venom of *V. berus* allowed obtaining several fractions eluted at different concentrations of NaCl: 0.1; 0.2; 0.3; 0.5 M. Non-binded fraction was also collected. The results of analysis of their action on compounds of the blood coagulation system are presented on Table.

Discussion. We assumed that in the case of *V. berus* and *V. nikolskii* the fibrinogenolytic action is masked by procoagulant components. However action on fibrinogen by the components of studied venoms cannot be neglected. Also we have to keep in mind that several purified snake venom proteins have become significant devices in fundamental exploration and in diagnostic procedures in hemostasiology. That is why further studies of fibrinogen-specific proteases of these species' venoms are promising. We also obtained fibrinogen-specific protease from *V. berus* venom. Its action substantially decreased the fibrinogen polymerization and also disrupted the ability of fibrinogen to support platelet aggregation.

Conclusions. Thus, the components of *Vipera* venoms living in Ukraine can be used for basic biochemical research. At the same time, care should be taken in the case of envenomation, as the presence of fibrinogenolytic enzymes in the venom can lead to hemorrhage. Further characterization of fibrinogen-specific protease from *V. berus* venom is a promising task for biotechnology.

Table. **Results of the analysis of fractions' properties of venom of Vipera berus berus. NB is the fraction that did not bind to the Q-sepharose under present conditions**

	Prolongation of the clotting time of plasma	Hemolysis of red cells	Activation of platelets	Inhibition of platelet aggregation
N.B.	+	–	+	–
0.1	–	–	+	–
0.2	+	–	–	+
0.3	–	+	–	–
0.5	–	+	–	–

Note. 0.1; 0.2; 0.3; 0.5 — fractions eluted at a NaCl concentration of 0.1; 0.2; 0.3; 0.5 M.

Key words: snake venom, *Vipera renardi*, *Vipera berus nikolskii*, *Vipera berus berus*, fibrinogenolytic action, fibrinogen-specific protease, APTT, enzyme-electrophoresis.

Funding source. The research was financed by the project 0119U002512: "The interaction of the hemostasis system components at the cellular and molecular level in the process of formation and elimination of a thrombus".

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UDC 577.112.7:577.121 https://doi.org/10.15407/biotech15.02.058

ADAPTOR PROTEIN RUK/CIN85 PARTICIPATES IN THE METABOLIC CONTROL OF HUMAN BREAST ADENOCARCINOMA MCF-7 CELLS

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Received 25.03.2022
Revised 21.04.2022
Accepted 29.04.2022

Cancer cells are able to dynamically change their metabolism to promote survival, maintain proliferative activity, provide high migration and invasive potential and increase metastasis. A common feature of this altered metabolism is increased glucose uptake and its fermentation to lactate, a phenomenon known as the Warburg effect [1].

Aim. To determine the role of Ruk/CIN85 in the control of breast adenocarcinoma cells metabolism, we performed systemic analysis of the activity levels/content of key enzymes/components of glycolysis and oxidative phosphorylation using as a model the weakly invasive human breast adenocarcinoma MCF-7 cell line (Mock); and its sublines with stable overexpression (G4 subline) and reverse down-regulation (G4vir subline) of the adaptor protein [2].

Materials and Methods. MCF-7 cells were cultured in the complete DMEM medium under standard conditions. Enzymes activity, content of metabolites and protein in cell extracts and the conditioned cell culture medium were estimated by spectrophotometric and fluorometric assays. The data obtained were analyzed with parametric Student`s t-test. Results were expressed as mean ± SEM and significance was set at $P < 0.05$.

Results and Discussion. First of all, biochemical indexes of aerobic glycolysis, activity levels of some key glycolytic enzymes and metabolites were evaluated [3]. A significant increase in the activity of these enzymes, aldolase A (ALDOA) and lactate dehydrogenase A (LDHA), was found in G4 cells compared to Mock by 1.3 and 1.6 times, respectively (Fig. *A*, *B*). In addition, in the conditioned medium of G4 cells, an increase in lactate content by 1.5 times compared with the control was found, which corresponded to a change in LDHA activity (Fig. *C*). Knockdown of Ruk/CIN85 expression level in G4 subline resulted in a significant decrease of these parameters compared to G4 cells, ALDOA — 4 times, LDHA — 1.4 times, and lactate production — 2.5 times. It should be noted that in G4vir cells, LDHA activity returned to level of control cells, while ALDOA activity and lactate content additionally decreased by 3 times and 1.6 times, respectively. Therefore, the observed changes in the intensity of glycolysis in MCF-7 sublines positively correlate with the expression level of adaptor protein studied.

To assess the metabolic status of mitochondria, the level of activity of the Krebs cycle enzyme, NAD-dependent malate dehydrogenase (MDH2), the catalyst of last stage of the cycle, was determined [3]. A 2-fold decrease in MDH2 activity was found in the MCF-7 G4 subline relative to control Mock cells, as well as an increase in this index by 2.4 times in G4vir cells to control values (Fig. *D*). Unlike glycolysis, we observed the opposite pattern with respect to the intensity of Krebs cycle reactions depending on the expression level of Ruk/CIN85.

Conclusions. The observed reversion of the Warburg metabotype as a result of Ruk/CIN85 down-regulation in MCF-7 cells overexpressing adaptor protein is a strong experimental evidence for its regulatory role in energy supply modes, aerobic glycolysis versus OXPHOS in the course cancer cells malignization.

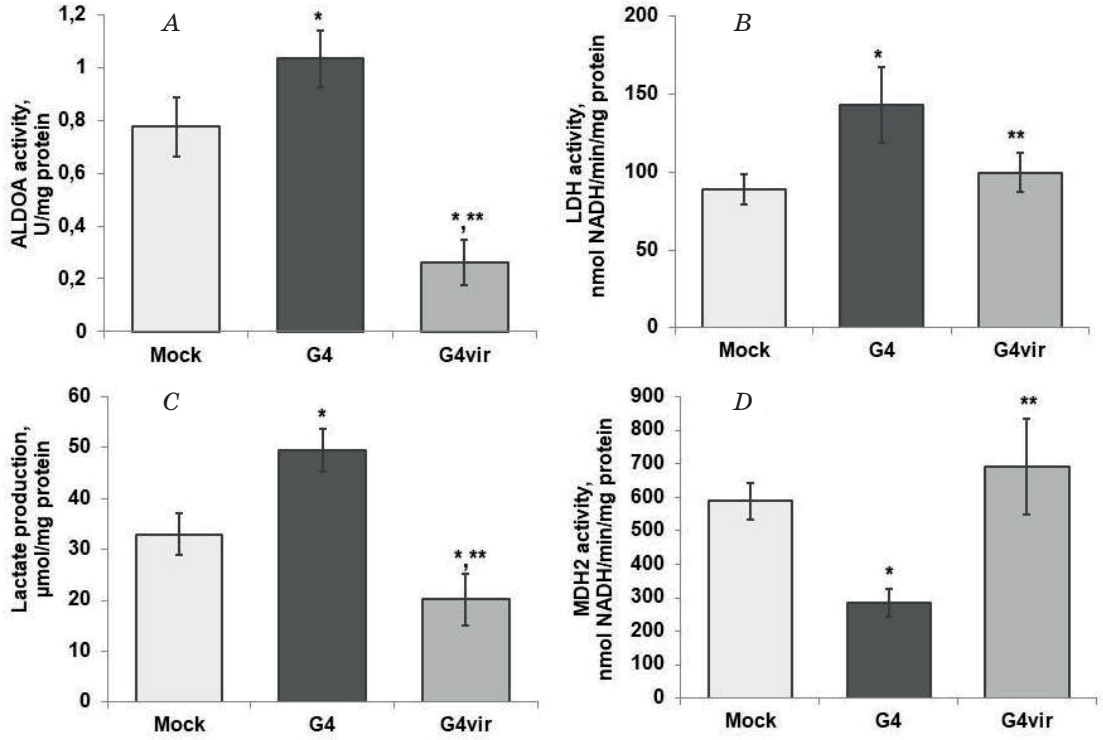

Fig. **Adaptor protein Ruk/CIN85 modulates metabolic indexes associated with Warburg effect in MCF-7 cells depending on its expression level**

Activities of enzymes: ALDOA (*A*), LDHA (*B*), MDH2 (*D*) in the cell extracts and lactate content in the conditioned cell culture medium (*C*), $M \pm m$, $n = 3$, * $P < 0.05$ to Mock, ** $P < 0.05$ to G4.

Funding

The present study was supported by the National Research Foundation of Ukraine for R&D "Differential control of regulatory networks involved in the maintaining of epithelial-mesenchymal plasticity of breast adenocarcinoma cells" (No 0120U104156).

Key words: breast adenocarcinoma cells, adaptor protein, Ruk/CIN85 Warburg effect, aerobic glycolysis, oxidative phosphorylation, aldolase A, lactate dehydrogenase A, lactate, NAD-dependent malate dehydrogenase.

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UDC 577.29 https://doi.org/10.15407/biotech15.02.060

LIMITED PROTEOLYSIS OF FIBRINOGEN αC-REGION REVEALS ITS STRUCTURE

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Received 25.03.2022
Revised 07.04.2022
Accepted 29.04.2022

αC-regions of fibrinogen (Aα392-610) are distant C-terminal parts of Aα-chains. Their three-dimensional structure is not clear because of high lability [1]. αC-regions cannot be crystallized properly that persuade scientists to search alternative ways of its structure analysis. It is known that proteolytic sites are located in the fragments of polypeptides that have no stable structure. So protease would rather cleave peptide bonds located in unstructured portions of polypeptide and bonds located between parts with distinct structure. That is why limited proteolysis of polypeptides allows to predict peculiarities of protein structure and to verify the three-dimensional computer models [2].

Aim. The purpose of our study was to compare hydrolytic action of proteases from *Gloydius halys halys Agkistrodon contortrix contortrix* and *Calloselasma rhodostoma rhodostoma* snake venoms and from *Bacillus thuringiensis* вар. *israelensis* IMV B-7465 culture medium on αC-regions of fibrinogen molecule.

Methods. Products of hydrolysis were characterized by SDS-PAGE under reducing conditions with following Western-Blot using the mouse monoclonal 1-5A (anti-Aα509-610) and II-5C (anti-Aα20-78) antibody. MALDI-TOF analysis of fibrinogen hydrolysis products was performed using a Voyager-DE.

Results. Combination of SDS-PAGE, FPLC and MALDI-TOF analysis allowed us to detect the peptide bonds cleaved by studied proteases. In particular proteases from *Gloydius halys halys* and *Agkistrodon contortrix contortrix* snake venoms cleaved peptide bond Aα413-414. Action of protease from *Calloselasma rhodostoma rhodostoma* on fibrinogen led to the formation of hydrolytic product generated from C-terminal portion of Aα-chain that corresponded to fragments generated by enzymes from two other snakes. On the other hand protease from *Bacillus thuringiensis* вар. *israelensis* IMV B-7465 culture medium cleaved peptide bond Aα504-505.

Discussion. The specificity of three unlike proteases from different species of snakes towards one particular peptide bond (Aα413-414) indicates that this fragment of αC-regions of fibrinogen

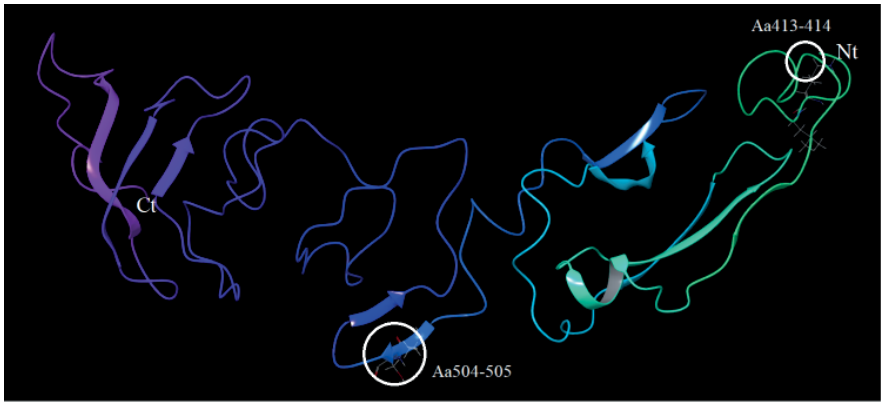

Fig. **Three-dimensional structure of fibrinogen αC-region with marked proteolytic sites.** Nt — amino-terminus; Ct — carboxy terminus part.

molecule most likely has no secondary structure. Also we can predict that fragments located on both sides of the polypeptide chain from Aα504-505 peptide bond must be exposed for the proteolytic action. These facts have to be acknowledged during 3D-modeling of αC-regions.

It is also notable to say that preliminary data indicate the presence of hydrolytic sites within 414-504 fragment of Aα-chain of fibrinogen. In particular such specificity was reported for fibrinogenase from *Echis multisquamatis* venom [3] and fibrinogen-specific protease from *Brahypelma smithi* venom [4]. This fact allows us to conclude the presence of proteolytic site that also can be exposed to the protease or located within a loop of polypeptide chain. Determination of accurate specificity of these proteases will allow to clarify the location of such unstructured sites of the αC-regions. Most likely such unstructured sites are surrounded by parts of molecule that have distinct structure.

Conclusions. Use of limited proteolysis technique as the source of additional information for computer modeling allowed us to propose an improved model of 3D-structure of fibrinogen αC-regions (Fig. 1). This model takes into account the behavior of αC-regions in the physiological condition and contributes to the general knowledge about fibrinogen structure.

Key words: αC-region, fibrinogen, C-terminal, proteases.

Acknowledgement. Authors gratefully acknowledged Dr Chernyshenko for his encouragement of these studies.

Funding source. This work was financed by the Palladin Institute of biochemistry of the National Academy of Sciences of Ukraine. PhD-program.

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UDC 616.89:615.099:599.323.4:612.08 https://doi.org/10.15407/biotech15.02.062

INFLUENCE OF SYSTEMIC INFLAMMATORY RESPONSE SYNDROME ON THE DEVELOPMENT OF OXIDATIVE STRESS DURING SIMULATION OF CHRONIC ALCOHOL INTOXICATION IN RATS

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Received 02.03.2022
Revised 27.03.2022
Accepted 29.04.2022

Sepsis is an important predictor of mortality among patients with alcoholic liver disease. About 60% of patients with alcoholic hepatitis have signs of systemic inflammatory response syndrome (SIRS) [1]. Increased susceptibility to bacterial infections in patients with severe alcoholic hepatitis is due to endotoxin-induced inhibition of neutrophils and lymphocytes [2]. Another important pathogenetic mechanism for the development of complications of alcoholic hepatitis is the penetration of lipopolysaccharide (LPS) through the intestinal barrier [3]. LPS through TLR4-dependent cascade stimulates Kupffer cells to release reactive oxygen species and a number of pro-inflammatory cytokines [4]. Ethanol metabolism also leads to the formation of reactive oxygen species. Chronic alcohol abuse and LPS can lead to free radical liver damage, but the question of their combined effects on the liver remains unclear.

The *aim* of our study was to analyze changes in the development of oxidative stress in the liver of rats with chronic alcohol intoxication against the background of systemic inflammatory response

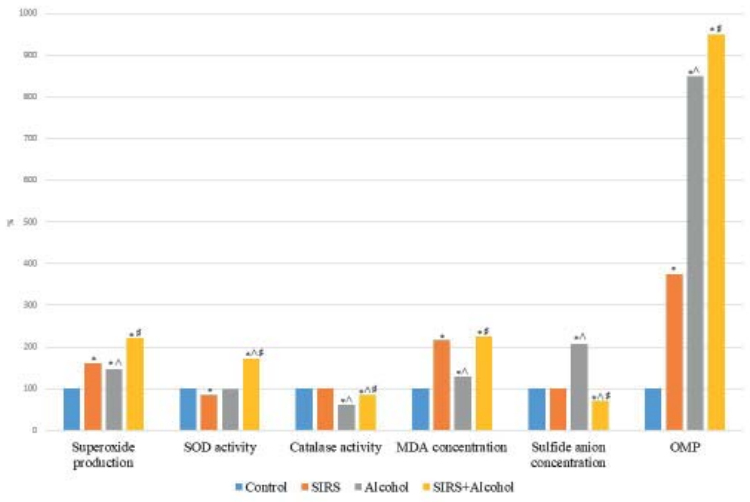

Fig. **Changes in the liver of rats under the conditions of modeling alcohol intoxication on the background of systemic inflammatory response syndrome**

* — $P < 0.05$ in comparison to the control group, ^ — $P < 0.05$ in comparison to the SIRS group, # — $P < 0.05$ in comparison to the alcohol group.

syndrome based on the study of catalase and superoxide dismutase activity, concentration of malonic dialdehyde, oxidatively modified proteins and sulfide anion and superoxide anion production.

Methods. Experimental studies were performed on 12 male Wistar rats weighing 180–220 g. Animals were divided into two groups: 1 — control and 2 — animals, on which we simulated alcoholic hepatitis and SIRS [4]. The activity of catalase (Korolyuk MA, 1988) and superoxide dismutase (SOD) (Brusov OS, 1976), the concentration of malonic dialdehyde (MDA) (Gérard-Monnier D, 1998), oxidatively modified proteins (OMP) (Dubinina OY, 2001), sulfide anion (Sugahara S, 2016) and superoxide anion production were studied in the rat liver homogenate (Kostenko VO, 2000). The obtained results were subjected to statistical processing using the Mann-Whitney test.

Results. Analyzing the development of oxidative stress in the liver of rats, on which we simulated the combined effects of SIRS and prolonged alcohol intoxication, we found that the activity of SOD increased by 1.72 times ($P < 0.05$), and catalase decreased by 1.18 times ($P < 0.05$) compared with the control group (Figure). The production of superoxide anion radical in the liver of rats increased 2.21 times ($P < 0.05$) in the group of animals with combined exposure to bacterial LPS and alcohol intoxication compared to control. The concentration of MDA increased 2.25 times ($P < 0.05$), and OMP by 9.5 times ($P < 0.05$) compared with control group. The concentration of sulfide anion in the liver of rats under the conditions of modeling the combined effects of SIRS and alcohol intoxication decreased by 1.44 times ($P < 0.05$) compared with the control.

Discussion. Stimulation of rat organism with bacterial LPS on the background of excessive alcohol intake leads to increase of superoxide production against the background of antiradical protection imbalance, accompanied by lipid peroxidation and oxidation of proteins.

Hydrogen peroxide, which is excessively formed under the action of SOD and is not completely utilized by catalase, reacts with ions of metals of variable valence, forming highly reactive hydroxyl radicals, which are powerful initiators of lipid peroxidation. Lipid peroxidation is the most important reaction involved in alcohol-induced liver damage through the formation of toxic aldehydes, including MDA. Acetaldehyde is also able to promote the formation of cross-links between DNA strands and create links between proteins and DNA, which causes replication and mutation errors in oncogenes or oncosuppressor genes with genotoxic, mutagenic and carcinogenic effects [3]. Given the powerful antioxidant properties of sulfide anion, a decrease in its concentration under the combined effects of chronic alcohol intoxication and stimulation of the organism with LPS may indicate depletion of the antioxidant potential of the liver.

Conclusions. Modeling of alcohol intoxication against the background of systemic inflammatory response syndrome leads to oxidative damage to lipid and protein structures of the liver due to increased production of superoxide anion radical and imbalance of antiradical protection.

Key words: alcoholic hepatitis, systemic inflammatory response syndrome, oxidative stress.

The authors state that they have no conflict of interest.

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UDC 577.112 https://doi.org/10.15407/biotech15.02.064

THE ACQUISITION OF RESISTANCE IN HUMAN NON-SMALL LUNG ADENOCARCINOMA MOR CELLS IS ASSOCIATED WITH UP-REGULATION OF ADAPTOR PROTEIN RUK/CIN85 AND EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT)

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Received 22.03.2022
Revised 17.04.2022
Accepted 29.04.2022

Non-small-cell lung carcinoma (NSCLC) is the most common type of cancer and cancer-related death in the world. High resistance of NSCLC to traditional chemotherapy is a serious problem for the effectiveness of treatment. Ruk/CIN85 is an adaptor/scaffold protein involved in the processes of tumor cells malignant transformation. By modulating protein-protein interactions in space and time, it potentially regulates signaling networks that control EMT-related cell responses such as proliferation, migration, invasiveness, and metastasis in the course of cancer progression [1].

The aim of this study was to elucidate the regulatory role of Ruk/CIN85 in chemoresistance and EMT using human NSCLC MOR cells as a model [2].

Methods. MOR (ECACC 84112312) cell line and drug-resistant cell line MOR/0.2R (ECACC 96042335) were cultured under standard conditions in DMEM medium. Knockdown of Ruk/CIN85 in MOR/0.2R cells was performed using shRNA lentiviral technology. Expression levels of Ruk/CIN85, vimentin and E-cadherin were estimated by RT-PCR.

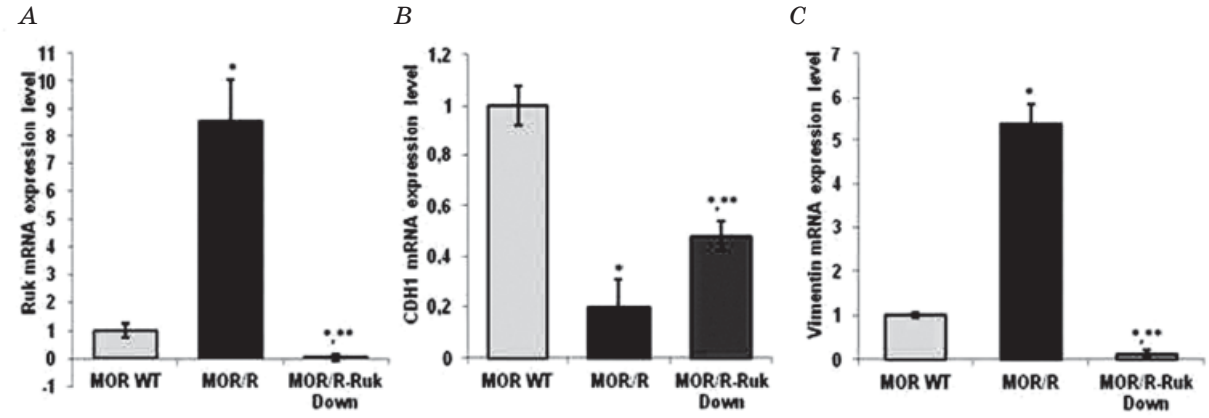

Fig. **Knockdown of Ruk/CIN85 in NSCLC MOR doxorubicin-resistant cells induces mesenchymal-epithelial transition:**
A, B, C — relative Ruk/CIN85, CDH1 and vimentin expression levels, respectively. (MOR WT — parental cells; MOR/R — drug-resistant MOR cells; MOR/R-Ruk Down — resistant MOR cells with Ruk/CIN85 knockdown); $M \pm m$, $n = 3$, * $P < 0.05$ to MOR WT, ** $P < 0.05$ to MOR/R

Results and Discussion. Taking into account the available data that up-regulation of Ruk/CIN85 in breast adenocarcinoma cells is followed by increase in their chemoresistance [3], we first compared the adaptor protein expression levels in MOR and MOR/0.2R cells (doxorubicin selected cells). According to the results of qPCR, MOR/0.R cells showed an extremely higher level of Ruk/CIN85 mRNA expression, more than 10 times higher than the parental MOR cells (Fig. *A*). These results were supported by data of Western-blotting. Also, preliminary data obtained in the Department of Cell Signaling revealed that knockdown of Ruk/CIN85 in the MOR/0.2R cells led to significant decrease of their resistance to doxorubicin and development of epithelial phenotype. So, in order to study the role of Ruk/CIN85 in EMT, we decided to check the expression levels of EMT epithelial marker E-cadherin as well as mesenchymal marker vimentin [4] in MOR sublines depending on adaptor protein expression levels. As can be seen from Figure, high content of RukCIN85 in doxorubicin-resistant (MOR/R) cells strongly correlate with their mesenchymal phenotype (high expression level of vimentin and low — E-cadherin), while its down-regulation is followed by restoration of expression values characteristic of parental MOR cells.

Conclusions. In summary, high expression level of Ruk/CIN85 in doxorubicin-resistant MOR cells and the reversion of EMT-related transcriptome parameters and sensitivity to drug due to knockdown of adaptor protein in this subline suggests its involvement in regulation of EMT as well as cancer cells chemoresistance. Thus, the adaptor protein Ruk/CIN85 can be considered as a tissue-specific marker of carcinogenesis and perspective target for drug development.

Key words: NSCLC, adaptor proteins, Ruk/CIN85, chemoresistance, epithelial-mesenchymal transition.

The authors state that they have no conflict of interest.

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UDC 577.115+57.084+57.037+616-006+615.03

https://doi.org/10.15407/biotech15.02.066

EFFECT OF N-STEAROYLETHANOLAMINE ON THE LIPID COMPOSITION OF THE FRONTAL CORTEX AND HIPPOCAMPUS OF THE RAT'S BRAIN AT THE AGING

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Received 20.03.2022
Revised 27.04.2022
Accepted 29.04.2022

Brain aging is an integral part of biological aging and is one of the most pressing medical and social issues today [1]. Lipids act as important regulators of various biological processes, including aging of the brain. Lipids play an important role in ensuring the structure of the brain and the regulation of its work. With age, the human brain decreases in most classes of lipids and fatty acid composition of brain tissues [2, 3].

Aim. It is important to study the possible protective effect of cannabimimetic lipid — N-stearoylethanolamine (NSE) on the lipid composition of the frontal cortex, hippocampus and on the state of episodic memory of old rats.

Methods. Experimental models on animals: the white outbred male 18-month-old rats (n=20) were used to investigate the influence of NSE treatment on the lipid composition of brain structures and young animals served as a control group. The water suspension of NSE at a dose 50 mg/kg was administered to old rats.

Extraction of lipids from the tissues of the hippocampus and frontal cortex of rats was performed by the method of Bligh and Dyer. Phospholipids were separated by two-dimensional thin layer chromatography. Methyl esters of fatty acids from lipid extract were obtained by a modified method of Carreau and Dubaco. Quantitative analysis of fatty acid methyl esters was performed by gas-liquid chromatography on an Agilent GC7890 chromatograph with an Agilent 8987 mass detector. The fractions of free and esterified cholesterol were separated by one-dimensional thin layer chromatography. The dry cholesterol residue was analyzed on a Carlo Erba gas-liquid chromatograph. Behavior test "New object recognition" Animal testing was performed twice — the first test was performed before the animals were divided into experimental groups, the second test was performed after the introduction of NSE. Statistical analysis was performed using Student's t-test, data at $P < 0.05$ were considered reliable.

Results. The study of the diacyl (DF) and plasmalogen (PF) forms of phospholipids (PLs) content in the frontal cortex and hippocampus have shown a significant decrease in the plasmalogen form of PE (Phosphatidylethanolamine) (up to 15%) and an increase in its DF, compare to its content in young rats. Administration of NSE to old rats led to a significant increase in PF PE and didn't cause significant changes in the content of PF in the composition of other PL of the frontal cortex of the brain and hippocampus (Table). The decrease in the percentage of various phospholipids was found in frontal cortex and hippocampus of old rats: the content of phosphatidylcholine (PC) and phosphatidylinositol (PI) was significantly reduced in the frontal cortex and the decrease of diphosphatidylglycerol (DPG), PI and phosphatidylserine (PS) was found in the hippocampus, compare to the young animals.

Administration of NSE to old rats had a different effects on the content of various phospholipids. The increase in the content of PC and PI in the frontal cortex and PS and DPG in the hippocampus is particularly pronounced due to NSE. It is known that DPG is a necessary PL for the functioning of the electron transport chain of mitochondria, and, consequently, to maintain cell energy (Table).

An increase in the content of saturated fatty acids (FFAs) and a decrease in the content of unsaturated FFAs in the frontal cortex and hippocampus of old rats also has been found. In particular, significantly reduced the content of polyenoic and monoenoic FFAs. The NSE administrations led to reducing the content of saturated FFA, normalizing the content of unsaturated FFA by increasing mono- and polyenoic FFA.

It has also been found that NSE administration to old rats promoted the growth of the free cholesterol level in the frontal cortex and hippocampus (Table).

Table. Effect of N-stearoylethanolamine on the lipid composition of the frontal cortex and hippocampus of the rat's brain at the aging, and discrimination rate of the "New object recognition" test at the aging

Indicators	Control group	Old rats	Old rats + NSE
Frontal cortex			
PC DF (%)	70 ± 1.13	94.45 ± 1.51 *	93.48 ± 1.24 #
PC PF (%)	30 ± 1.13	5.55 ± 1.51*	6.52 ± 1.24 #
PE DF (%)	39.55 ± 1.38	88.20 ± 1.84 *	56.01 ± 5.22 #
PE PF(%)	60.45 ± 1.38	11.80 ± 1.84 *	43.99 ± 5.22 #
PC (%)	34.64 ± 0.38	31.59 ± 1.35 *	36.26 ± 0.81 #
PI (%)	7.5 ± 0.36	3.87 ± 0.16 *	7.27 ± 0.44 #
Cholesterol (μg/g lipids)	5000 ± 500	3500 ± 350 *	4500 ± 580 #
Hippocampus			
PC DF (%)	71.94 ± 0.92	95.93 ± 1.23 *	92.35 ± 0.16 #
PC PF (%)	3.06 ± 0.92	4.07 ± 1.23 *	7.65 ± 0.16 #
PE DF (%)	35 ± 1.51	57.24 ± 2.01 *	49.35 ± 2.80 #
PE PF (%)	65 ± 1.51	42.76 ± 2.01 *	52.89 ± 2.89 #
DPG (%)	3.46 ± 0.26	2.65 ± 0.01 *	3.84 ± 0.46 #
PI (%)	9.21 ± 0.6	6.58 ± 0.03 *	6.9 ± 0.89 #
PS (%)	15.67 ± 0.32	10.14 ± 0.58 *	12.39 ± 0.25 *#
Cholesterol (μg/g lipids)	3000 ± 500	2500 ± 350 *	2600 ± 580 #
Discrimination index of the "New Object Recognition" test	0.376667 ± 0.02	0.20598 ± 0.035 *	0.296624 ± 0.03 #

Notes: PF — plasmalogen form, DF — diacyl form, PC — phosphatidylcholine, PE – phosphatidylethanolamine, PI — phosphatidylinositol, PS — phosphatidylserine, DPG — diphosphatidylglycerol, % — of the total amount of phospholipids, * — $P < 0.05$ compared to the group "Control group", # — $P < 0.05$ compared to the group "Old Rats".

The results of the New Object Recognition test in old rats have shown that a short-term memory has been improved by NSE (Table).

Conclusions. The administration of NSE to old rats causes an increase in PF of PLs in the frontal cortex and hippocampus of the brain, which can be considered as one of the mechanisms of neuroprotective action of NSE in aging. The changes in the phospholipids and fatty acids composition, and free cholesterol level of the frontal cortex and hippocampus of the brain of old rats caused by NSE administration have been shown to be adaptive and restorative. The New Object Recognition Behavioral Test has shown that NSE restores short-term memory in older rats.

The obtained results expand the understanding of the mechanisms of biological action of NSE during aging in mammals and create the basis for the development a new drug with geroprotective properties.

Key words: N-stearoylethanolamine, aging, phospholipids, plasmalogens, fatty acids, cholesterol, memory.

Acknowledgement. The authors gratefully acknowledge Ph.D. Kosiakova H.V., Ph.D. Berdyshev A.G., Ph.D. Meged' O.F., Ph.D. Klimashevsky V.M.

The authors state that they have no conflict of interest.

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UDC 577.112.7:616 https://doi.org/10.15407/biotech15.02.068

GRAPHENE OXIDE AFFECT THE EXPRESSION OF PROLIFERATION RELATED GENES AND MICRORNA IN NORMAL HUMAN ASTROCYTES

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Received 29.03.2022
Revised 16.04.2022
Accepted 29.04.2022

Graphene and its oxide are nanomaterials, which have the exceptional physicochemical properties considered currently to be very promising because of their great potential applications in various industries. Graphene oxide, an oxidation derivative of graphene, is considered as one of the nanomaterials attractive for biomedical applications in the fields of drug delivery, cancer therapy, and diagnostics [1–3]. The increasing exploitation of graphene-based materials requires a comprehensive evaluation of the potential impact of these unique carbon nanoparticles on human health and the environment [4]. At the same time, potential risks have also been recognized, as the hazardous impact of various carbon nanoparticles on human health and the environment was previously shown [5–6]. Thus, the toxic potential of carbon nanoparticles was reported in numerous cell lines and animal models and their long-term toxicity has attracted increasing concern.

Aim. In this study we investigate the impact of low doses of graphene oxide on the expression of key regulatory genes which control cell proliferation as well as microRNAs in normal human astrocytes.

Methods. The expression level of genes related to cell proliferation was studied by real-time qPCR in normal human astrocytes line NHA/TS (Cambrex Bio Science, Walkersville, MD, USA) using SYBRGreen Mix and specific for each mRNA forward and reverse primers as described [7]. These astrocytes were treated with graphene oxide (1 and 4 ng/ml of medium) for 24 hrs. Graphene oxide (2 mg/ml, dispersion in water) was received from Sigma-Aldrich Chemie GmbH, Germany. Total RNA was extracted using TRIZOL reagent. For reverse transcription of mRNAs we used Thermo Scientific Verso cDNA Synthesis Kit (Germany). The values of mRNA expressions were normalized to the level of ACTB mRNA and represented as percent of control (100%). For polyadenylation and reverse transcription of miRNAs we used Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan). The expression level of microRNAs was studied by real-time qPCR using SYBRGreen Mix and specific for each miRNA forward primers and universal reverse primer. For normalization of microRNA expressions the level of U6 RNA expression was used.

Results. It was shown that the expression level of *TOB1, HSPA5, EDEM1, MYBL1*, and *MYBL2* significantly increased in normal human astrocytes line NHA/TS, which were treated with graphene oxide (1 and 4 ng/ml of medium) for 24 hrs. Up-regulation of these genes expression was dose-dependent: bigger dose of graphene oxide (4 ng/ml of medium) introduced more significant changes in the expression of all these genes. Furthermore, bioinformatics analysis of 3′-untranslated regions of mRNA allowed identifying binding sites of microRNA: miR-19a for MYBL1, miR-143 for MYBL2 and miR-182 for TOB1. It was also shown that the expression of all these microRNA significantly down-regulated by graphene oxide, supporting the idea of both post-transcriptional and transcriptional regulation of *MYBL1, MYBL2* and *TOB1* gene expressions (Figure).

Discussion. Results of this study showed that graphene oxide affects the expression of *MYBL1, MYBL2,* and *TOB1* genes through both transcriptional and post-transcriptional mechanisms. It is possible that these changes in gene expressions are mediated by the endoplasmic reticulum stress, which strongly induces *HSPA5/GRP78* gene expression.

Conclusions. Graphene oxide significantly disturbs genome stability by up-regulation of the expression of key regulatory genes and down-regulation of microRNA.

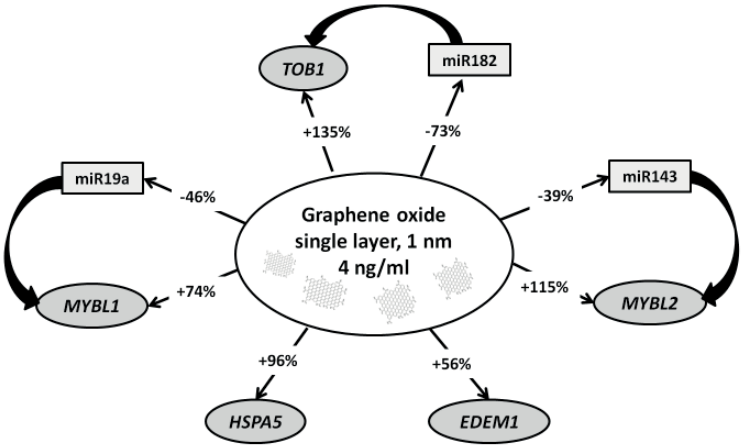

Fig. **The Impact of low dose of graphene oxide on the expression level of proliferation related genes and microRNAs in normal human astrocytes**

Key words: Graphene oxide, proliferation, gene expression, microRNA, normal human astrocytes.

Funding source. The work was done according the State Budget Program (Code: 6541230).

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UDC 577.112.616 https://doi.org/10.15407/biotech15.02.070

GLUTAMINE DEPRIVATION AFFECTS THE EXPRESSION OF GENES WHICH CONTROL PYRUVATE DEHYDROGENASE ACTIVITY: THE IMPACT OF ERN1 KNOCKDOWN

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Received 25.03.2022
Revised 08.04.2022
Accepted 29.04.2022

Pyruvate dehydrogenase (PDH) is a mitochondrial multienzyme complex that catalyzes the oxidative decarboxylation of pyruvate and is one of the major enzymes responsible for the regulation of homeostasis of carbohydrate fuels in mammals [1-2]. It is known that pyruvate dehydrogenase kinase inhibits pyruvate dehydrogenase activity via phosphorylation of its subunits PDHA1 and PDHA2 and thereby regulates metabolite flux through the tricarboxylic acid cycle, down-regulates aerobic respiration and inhibits the formation of acetyl-coenzyme A from pyruvate. Glutamine is a substrate for glycolysis and thereby is important for the development and a more aggressive behavior of malignant tumors, especially gliomas, which are highly aggressive tumors with very poor prognosis. It is well known that glutamine supply and endoplasmic reticulum stress are very important and complementary factors for the malignant tumor growth. Furthermore, inhibition of ERN1 (endoplasmic reticulum to nucleus signaling 1), a major signaling pathway of endoplasmic reticulum stress, significantly modifies the effects of glutamine deprivation on the expression of numerous genes [3]. At the same time, the comprehensive molecular mechanisms of the interaction of stress signaling pathway mediated ERN1 with glutamine supply are complex yet and warrant additional study. Moreover, there is also data that nutrient starvation is a very important factor of the resistance of cancer cells to chemotherapy. It is well known that activation of ERN1 branch of the endoplasmic reticulum stress response is tightly linked to apoptosis and cell death and that inhibition of its function has been demonstrated to result in a significant anti-proliferative effect in glioblastoma growth.

The aim of the current investigation was to study the expression of genes encoded pyruvate dehydrogenase subunits (PDHA1, PDHB, PDHX, DLAT, and DLD) in U87 glioma cells in response to glutamine deprivation in U87 glioma cells in relation to knockdown of ERN1 for evaluation of a possible dependence of the expression of these important regulatory genes from glutamine supply and ERN1 signaling.

Methods. The expression of *PDHA1, PDHB, PDHX, DLAT*, and *DLD* genes was studied by real-time qPCR in control U87 glioma cells (transfected by vector) and cells with knockdown of ERN1 (transfected by dnERN1) after exposure to glutamine deprivation condition. Total RNA was extracted from glioma cells using TRIZOL reagent. An RNA quantity as well as spectral characteristics was measured using NanoDrop One. For reverse transcription of mRNAs we used Thermo Scientific Verso cDNA Synthesis Kit (Germany). The values of mRNA expressions were normalized to the level of ACTB mRNA and represented as percent of control (100%).

Results. It was shown that the expression level of *PDH1, PDHB, DLAT*, and *DLD* genes was down-regulated in control glioma cells treated by glutamine deprivation. At the same time, ERN1 knockdown is suppressed the effect of glutamine deprivation on *PDHB* and *DLD* gene expressions in glioma cells, but did not change significantly the impact of glutamine deprivation on the expression of *PDHA1, DLAT*, and *PDHX* genes (Figure).

Discussion. These results are important for the evaluation of possible significance of glutamine deprivation in ERN1 dependent control of glioma cell proliferation because there are data indicating

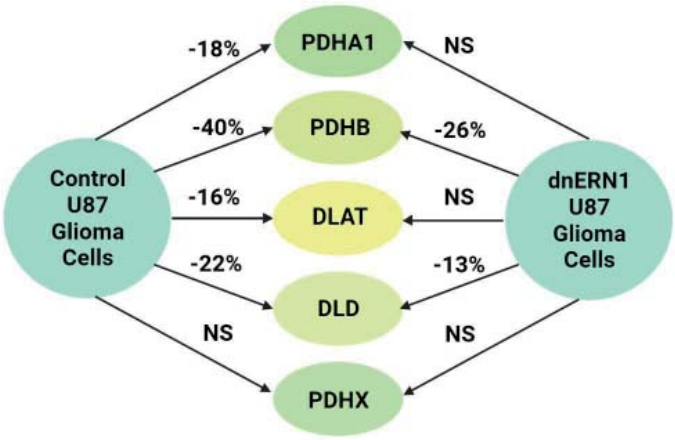

Fig. **The impact of glucose deprivation on the expression level of genes encoding pyruvate dehydrogenase subunits PDHA1, PDHB, DLAT, DLD, PDHX in control and ERN1 knockdown U87 glioma cells**

that the endoplasmic reticulum stress signaling mediated by ERN1 is involved in numerous metabolic pathways and ERN1 knockdown has clear anti-tumor effect [3]. Results of this study clarify possible mechanisms of glutamine deprivation on the proliferation/surviving of ERN1 knockdown glioma cells through specific changes in the expression profile of genes encoding subunits of PDH.

Conclusions. The results of this investigation demonstrated that the expression of *PDH1, PDHB, PDHX, DLAT,* and *DLD* genes was significantly affected by exposure of U87 glioma cells under glutamine deprivation condition and that the effect of glutamine deprivation on the expression of most these genes was modified in cells with knockdown of ERN1, a major signaling pathway of the endoplasmic reticulum stress.

Key words: pyruvate dehydrogenase, mRNA expression, ERN1 knockdown, glutamine deprivation, U87 glioma cells.

Funding source. The State Budget Program (Code: 6541230).

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UDC 577.112.616 https://doi.org/10.15407/biotech15.02.072

MOLECULAR BASIS OF THE DEVELOPMENT OF INSULIN RESISTANCE IN OBESE ADOLESCENT AND ADULT MEN

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Received 23.03.2022
Revised 07.04.2022
Accepted 29.04.2022

Obesity is a serious and urgent public health problem because the number of people including children with severe obesity is significantly increased. Furthermore, children with severe obesity are at greater risk for adult obesity, early atherosclerosis, hypertension, metabolic syndrome, type 2 diabetes, fatty liver disease, and premature death. The development of obesity and its metabolic complications is associated with dysregulation of various intrinsic mechanisms, which control basic metabolic processes, including cellular growth, glucose, and lipid metabolism as well as insulin sensitivity, via changes in the expression of numerous regulatory genes including genes related to glucose metabolism and their regulations [1-3]. Moreover, obesity and its complications result from interactions between genes and environmental factors and is associated with changes in gene expressions of regulatory network in various organs and tissues, but preferentially in adipose tissue. Therefore, adipose tissue growth is in a center of obesity and is tightly associated with the glucose and lipid metabolism as well as with cell proliferation processes and is controlled by different interconnected regulatory factors and enzymes. At the same time, the blood reflects numerous changes in different organs and tissues in diseases including obesity. Special interest deserves the key regulatory enzymes and factors, which control glucose and lipid metabolism as well as cell growth. Receptors of insulin-like growth factor (IGF) and insulin as well as related proteins play an important role in the regulation of numerous metabolic and proliferative processes and participate in endoplasmic reticulum stress, which is an important factor of obesity, insulin resistance, and tumor growth. Furthermore, there exists a cross talk between IGF and insulin receptor signaling pathways at the receptor level or downstream signaling level. A specific feature of obesity and associated insulin resistance, as well as a number of other pathological conditions, is impaired maturation of proteins in the endoplasmic reticulum and the accumulation of unfolded or improperly folded proteins, called endoplasmic reticulum stress. This stress is an important factor in the development of insulin resistance, as well as many metabolic complications in obesity, because the endoplasmic reticulum stress disrupts the signaling pathways from the insulin receptor. Therefore, endoplasmic reticulum stress is a factor that controls the expression of a large number of genes, including those that control glucose metabolism, and links obesity and its complications.

The aim of this work was to study the association between the expression of glucose metabolism related genes and insulin resistance, which expression is changed in obese adolescents and adult men with and without insulin resistance, for better understanding the molecular basis of the development of obesity complications and evaluation of possible contribution of these genes in development of insulin resistance.

Methods. The expression level of genes related to glucose metabolism and their regulations was studied by real-time qPCR in adipose tissue and blood cells using SYBRGreen Mix and specific for each mRNA forward and reverse primers. Total RNA was extracted using TRIZOL reagent. For reverse transcription of mRNAs we used Thermo Scientific Verso cDNA Synthesis Kit (Germany). The values of mRNA expressions were normalized to the level of ACTB mRNA and represented as percent of control (100%).

Results. It was shown that in obese patients with insulin resistance the expression level of IRS1 (insulin receptor substrate 1), *HK2* (hexokinase 2), *PFKFB2* (6-phosphofructokinase/fructose-2,6-bisphosphatase 2) and *PFKFB3* as well as circadian factors *CLOCK* and *ARNTL* genes in subcutaneous adipose tissue is significantly decreased as compared to obese men with normal sensitivity to insulin. At the same time, the development of insulin resistance in obese patients leads to up-regulation of *PFKFB4, PER1, HSPA6, ALDH1A3, COL5A1, TIMP1, TIMP2, SPARC*, and *VCAN* gene expressions in subcutaneous adipose tissue. The expression level of IGF1 (insulin-like growth factor 1) and *IGFBP5* (IGF binding protein 5) as well as *ENO1* (enolase 1) and *ENO2* is down-regulated in the blood of obese adolescent with insulin resistance, but *IGFBP2* and *IGFBP7* gene expressions are significantly increased in these patients.

Discussion. It is possible that the changes in the expression of *IRS1, IGF1, IGFBP2, IGFBP5*, and many other regulatory genes are mediated by the endoplasmic reticulum stress and contribute to the development of insulin resistance and glucose intolerance as well as to other complications [3].

Conclusions. The results of this investigation provide evidence that the development of insulin resistance in obese patients is associated with gene specific changes in the expression of many very important regulatory genes, which are endoplasmic reticulum stress responsible.

Key words: obesity; insulin resistance; genes; subcutaneous adipose tissue.

Funding source. The State Budget Program (Code: 6541230).

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UDC 577.112.7 https://doi.org/10.15407/biotech15.02.074

EPITHELIAL-MESENCHYMAL TRANSITION IN MELANOMA PROGRESSION: THE CONTRIBUTION OF ADAPTOR PROTEIN RUK/CIN85

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Received 07.04.2022
Revised 20.04.2022
Accepted 29.04.2022

Adaptor proteins of multi-modular structure are essential components of signaling networks. By interaction with other proteins, nucleic acids and lipids, they serve as frameworks for multimolecular complexes assembly [1]. It allows them to play a crucial role in signal transduction. Therefore, genetic alterations in the structure of such important cellular regulators and changes in their expression can lead to signaling disturbances, which may result in the emergence of numerous diseases, including cancer. Reversible process of epithelial-mesenchymal transition (EMT) has been recognized as driving force in metastasis of epithelial cell malignancies. The progression of melanoma, like that of carcinoma, is also characterized by a "phenotype switching" pattern reminiscent of EMT [2].

Aim. In light of the reported relationship between primary melanoma stages and the adaptor protein Ruk/CIN85 expression levels [3], the purpose of this study was to test the hypothesis that Ruk/CIN85 overexpression/knockdown in melanoma cells may be involved in the regulation of EMT.

Materials and methods. The mouse melanoma cell line B16-F10 and its sublines with up-/down-regulation of Ruk/CIN85 (generated early using lentiviral technology) were used as a model for research. Melanoma cells were cultured in the complete RPMI 1610 medium under standard conditions. Proliferative activity of the cells was estimated using the MTT-test, and cell migratory potential was studied by the wound-healing assay. The data obtained were analyzed with parametric Student`s t-test. Results were expressed as mean ± SEM and significance was set at $P < 0.05$.

Results and Discussion. Cutaneous melanoma genesis is a multi-step process initiated by the transformation of a normal melanocyte following an oncogenic insult. Due to the transcriptome and metabolome reprogramming in the course of EMT, transformed melanoma cells change their phenotype and acquire increased proliferative rate, cell motility, invasiveness, and metastatic potential. According to the data obtained, overexpression of Ruk/CIN85 in B16 mouse melanoma cells (subclones Up7 and Up21) led to an increase in their proliferative activity by 1,6 and 1,8 times, respectively, at 24th hour in comparison with control Mock cells (Fig. *A*). At the 48th hour, when the cells reached confluence, the cell viability of subclones did not differ from the control ones. No statistically significant changes in the proliferative activity of B16 cells with suppressed expression of the adaptor protein (subclone Down) were found. In accordance with previous data [4], B16 cells overexpressing Ruk/CIN85 were characterized by strongly increased motility rate (more than twofold for both Up7 and Up21 subclones compared to control Mock cells). At the same time, knockdown of Ruk/CIN85 in B16 cells resulted in a decrease in their migratory activity by about 30% (Fig. *B*).

Conclusions. All findings obtained demonstrated that the malignancy traits of melanoma B16 cells are inversely modulated upon up- and down-changes in adaptor protein Ruk/CIN85 expression levels suggesting its possible role in the control of EMT.

Key words: melanoma, adaptor proteins, Ruk/CIN85, epithelial-mesenchymal transition, proliferation, migration.

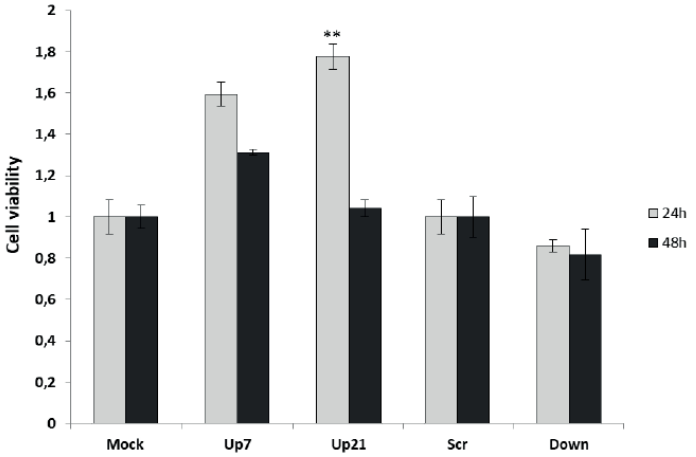

Fig. **Adaptor protein Ruk/CIN85 modulates viability and motility rate of mouse melanoma B16 cells depending on its expression level:**
A — cell viability; *B* — motility rate. (Up7, Up21 — subclones of B16 cells verexpressing Ruk/CIN85 and corresponding control Mock cells; Down — B16 cells with down-regulation of Ruk/CIN85 and corresponding control Scr cells).
$M \pm m$, $\boldsymbol{n} = 3$, *,** $P < 0.05$ to Mock and Scr.

The authors state that they have no conflict of interest.

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