Biannual edition
Languages of publication: English, Romanian, French, Russian
Founder: Moldavian Biosafety and Biosecurity Association

ISSN 2587-3458 (Print)
e-ISSN 2587-3466 (Online)
Edited by: Typography "Print-Caro", **Edition:** 300 ex.
Registered at the Ministry of Justice with no. 476676, 5th of July, 2017

COVID-19 – Pandemic Potential Infecting Humans – Call for a One Health Approach

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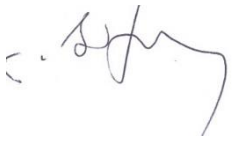
Today, when the world faces complex problem - COVID-19 pandemic, no single discipline, institution, or country can respond to it alone. Covid-19 is closely related to viruses that infect bats, however, the animal species that acted as the intermediate host to enable the disease spread onto humans is still unknown.

60% of infectious diseases that infect humans come from animals. Many medical experts believe we should be taking preventative measures to help avoid another outbreak by adopting the "One Health" approach. As scientists continue to search for the mystery animal, how is it possible to prevent a future pandemic if the origins of the novel coronavirus remains unclear?

One Health, which is advocated by the World Health Organization, is designed to implement policies and programs where a range of professionals and experts like vets, doctors and environmental scientists can join forces and share data to help avert another pandemic. One Health strategy would be able to predict where outbreaks might occur by surveilling places where humans and animals come into frequent contact, such as live animal markets, meat processing centers and heavy poultry markets.

Therefore, I wish OH&RM to review COVID-19 outbreak in a one health context, highlighting the need for the implementation of one health measures and practices to improve human health and reduce the emergence of pandemic viruses.



OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

SYNTHESIS ARTICLE – ARTICOLE DE SINTEZĂ – ARTICLES DE SYNTHÈSE – ОБЗОРНЫЕ СТАТЬИ

MEDICAL SCIENCES

OPEN ACCESS

THE WORLD-WIDE SPREAD OF CARBAPENEM-RESISTANT ENTEROBACTERALES

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DOI: 10.38045/ohrm.2021.1.01 UDC: 579.842.16:615.281.9.015.8

Key words: *carbapenem resistant Enterobacterales, carbapenem-resistant Klebsiella pneumoniae, carbapenems, carbapenemases.*

Introduction*. Gram-negative bacilli belonging to the order Enterobacterales are normal inhabitants of the human gut, which also are the most common causative agents of both nosocomial and community acquired infections in patients of all ages. Although not even a century has passed since Fleming's discovery of penicillin, the scientists have been alarmed by the fact that the "last resort antibiotics" viz. carbapenems have been compromised.* ***Material and methods.*** *The analysis of fifty-two articles and documents regarding this topic was peformed.* ***Results.*** *The main mechanism of resistance to carbapenems in Enterobacterales is production of carbapenemases, being enzymes that destroy all or almost all β-lactam antibiotics including carbapenems. According to Ambler's classification β-lactamases can be distributed into four classes (A, B, C, and D) being based on primary amino acid sequence homology. The most important carbapenemases produced by Enterobacterales belong to class A (KPC), class B (metallo-β-lactamases NDM, VIM, IMP) and class D (OXA-48-like). Unlike other mechanisms of resistance, carbapenemase production is easily spread via plasmids making carbapenemase-producing Enterobacterales (CPE) a global challenge for healthcare providers.* ***Conclusions.*** *CPE are not readily detected in the laboratory but the ability to detect carbapenemase production in Enterobacterales has very important infection control implications and therefore is essential for local infection control programs and national and international surveillance systems. Furthermore, local epidemiology of multidrug resistant organisms has major influence on development of national clinical guidelines for antimicrobial use.*

Cuvinte cheie: *Enterobacterales carbapenem rezistente, Klebsiella pneumoniae carbapenem-rezistentă, carbapeneme, cabapenemaze.*

RĂSPÂNDIREA MONDIALĂ A ENTEROBACTERALES CARBAPENEM-REZISTENTE

Introducere. *Bacilii gramnegativi din ordinul Enterobacterales habitează la nivelul intestinului uman, dar în același timp sunt și cei mai comuni agenți cauzali ai infecțiilor nozocomiale și comunitare la pacienții de toate vârstele. Deși nu a trecut nici măcar un secol de la descoperirea penicilinei de către Fleming, suntem deja într-o situație îngrijorătoare în care „antibioticele de ultimă instanță", carbapenemele, au fost compromise.*

Material și metode. *Au fost analizate cincizeci și două de articole și documente pe tema analizată.* ***Rezultate.*** *Mecanismul principal de rezistență la carbapeneme la Enterobacterales este producerea enzimelor carbapenemaze, care distrug toate sau aproape toate antibioticele β-lactamice, inclusiv carbapenemele. Conform clasificării Ambler, β-lactamazele pot fi distribuite în patru clase (A, B, C și D) pe baza omologiei primare a secvenței aminoacizilor. Cele mai importante carbapenemaze produse de Enterobacterales aparțin clasei A (KPC), clasei B (metallo-β-lactamaze NDM, VIM, IMP) și clasei D (OXA-48-like). Spre deosebire de alte mecanisme de rezistență, producerea de carbapenemaze este ușor răspândită prin intermediul plasmidelor, făcând Enterobacterales (CPE) producătoare de carbapenemază o provocare globală pentru lucrătorii medicali.* ***Concluzii.*** *Nu este ușor de detectat CPE în laborator, dar abilitatea de a detecta producerea de carbapenemaze la Enterobacterales este foarte importantă în control infecției și, prin urmare, este esențială pentru programele locale de control al infecțiilor și sistemele de supraveghere naționale și internaționale. Mai mult, epidemiologia locală a organismelor multirezistente are o influență majoră asupra dezvoltării ghidurilor clinice naționale pentru utilizarea antimicrobienelor.*

INTRODUCTION

Enteric Gram-negative bacilli are important part of human microbiota and they used to be referred to as *Enterobacteriaceae*. However, with the new nomenclature this group of bacteria should be referred to as the order Enterobacterales which includes the family *Enterobacteriaceae* but also some other medically important families. This largest group of Gram-negative facultative anaerobes and non-spore-forming rods has a critical role in human medicine because its members are normal inhabitants of the human gut but at the same time also the most common causative agents of both nosocomial and community acquired infections in patients of all ages (1, 2). Occasionally, and mainly in people with underlying disease, these bacteria can invade the blood or tissues and cause serious infections that have been so far successfully treated with antibiotics.

Although they have been in clinical use for 75 years, beta-lactam antibiotics are the most commonly prescribed antimicrobial drugs because of their characteristics such as good safety profile (except for allergic reactions that occur rarely), high bactericidal activity, and broad spectrum (3). Carbapenems are broad spectrum β-lactam antibiotics, being highly effective against most Gram-negative infections even in cases when causative organisms are resistant to most other antibiotics. Wide use of carbapenems, even in situations when they were not needed, has led to the emergence of resistance to these valuable "last resort antibiotics". Carbapenem resistance can be mediated by different mechanisms such as the reduced cell wall permeability, hyperexpression of efflux pumps or production of enzymes that hydrolyze broad spectrum β-lactams including carbapenems viz. the carbapenemases. Carbapenemase production seems to be the most important resistance mechanism in Enterobacterales as it quite often confers high level of resistance and is easily spread *via* plasmids. Carbapenemase-producing Enterobacterales (CPE) are therefore the most important subset of carbapenem resistant Enterobacterales (CRE). Although many different bacterial species within Enterobacterales can acquire genes for carbapenemase production, of particular concern is the increasing carbapenem resistance in *Klebsiella pneumoniae* and *Escherichia coli* (4). An alarming issue is that some *K. pneumoniae* clones show particular potency for epidemic spread.

Rapid worldwide spread of CPE has become a global challenge for healthcare providers, making treatment of such patients a difficult task (5). The World Bank made an estimate that by 2050, 10 million people could die annually, because of infections caused by multidrug resistant organisms (MDROs) if no measures against antimicrobial resistance are implemented (6). Antimicrobial resistance has a direct impact on the success of infectious diseases treatment and prophylaxis and seriously jeopardizes advances in many areas of healthcare, increases mortality, prolongs stays in hospital, increases costs and is therefore recognized by World Health Organization (WHO) as a profound threat to human health (7). The indirect impact of antimicrobial resistance includes reduction in gross domestic product (GDP) caused by economic losses due to reduced productivity and higher costs of treatment which could cause global economic damage on a par with the 2008 financial crisis (8). In 2015, the study carried out by European Center for Disease Prevention and Control (ECDC), regarding the burden caused by infections with antibiotic-resistant bacteria on the European Union (EU) and the European Economic Are, estimated the highest burden of carbapenem-resistant *K. pneumoniae* (9). This study review provides an overview of the epidemiology of carbapenemase producing (CP) *K. pneumoniae*.

MATERIAL AND METHODS

Review article. A search of the literature was performed on the Internet using PubMed database, Google, Google Scholar by applying the following keywords: Enterobacterales, *Klebsiella pneumoniae*, carbapenems, beta-lactamases, carbapenemases, antimicrobial resistance surveillance. The final bibliography included 50 references.

RESULTS

Evolution of β-lactamases

Although Alexander Fleming began the antibiotic era with the discovery of penicillin in 1928, it started being widely produced and clinically used 12 years later thanks to Howard Florey and Ernest Chain (10). A broad-spectrum penicillin viz. the ampicillin, which is highly effective against some Enterobacterales was discovered 20 years later. Shortly after the introduction of ampicillin, the first enzymes that hydrolyze penicillins and early generation cephalosporins, such as

TEM-1 and SHV-1 were described and named broad-spectrum-β-lactamases (11). In the 1980s, as a response to bacteria producing broad-spectrum-β-lactamases, oxyimino-cephalosporins, the third generation cephalosporins such as cefotaxime, ceftriaxone and ceftazidime were introduced into clinical use. These drugs are poor substrates for the broad spectrum β-lactamases and show high bactericidal efficacy against a wide range of Enterobacterales (11, 12). Along with the development of new generations of cephalosporins, combinations of a β-lactam and a β-lactamase inhibitor, such as amoxicillin/clavulanic acid, ampicillin/sulbactam and piperacillin/tazobactam were developed to combat resistance mediated by broad-spectrum-β-lactamases (13). The wide use of the third generation cephalosporins placed selective pressure on bacteria resulting in the evolution of variants of broad-spectrum-β-lactamases that have gained the ability to hydrolyze oxyimino-cephalosporins and these enzymes were named extended-spectrum-β-lactamases (ESBLs) (14). Another resistance mechanism against the third generation cephalosporins includes hyperproduction of AmpC β-lactamase, which was first detected in bacterial species that have chromosomally encoded inducible AmpC β-lactamase (*Enterobacter* spp., *Citrobacter freundii*, and *Serratia* spp.) (15). Later, genes encoding for AmpC β-lactamase hyperproduction were transferred by plasmids to other bacterial species such as *K. pneumoniae* and *E. coli* (15). ESBL and AmpC producing isolates spread globally during 1980s and 1990s promoting the use of carbapenems, which are often referred to as „last resort antibiotics" (2, 16). Carbapenems have a broad antibacterial activity, since they are stable to hydrolysis by ESBL and AmpC enzymes and have a safe profile in terms of side effects. Carbapenems were reliable and highly effective solution for multi-resistant Gram-negative bacteria for over 15 years, however instead of being used cautiously when only a broad coverage was required, they were often used excessively in empirical therapy without identifying the bacterial pathogen or without de-escalating antibiotic therapy in case a bacteriological finding was available. Consequently, carbapenem resistance to Gram-negative bacteria, particularly in *K. pneumoniae*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* has spread and represents a major on-going public health problem worldwide. Carbanem resistance in *A. baumannii* is mostly mediated by plasmid- or chromosomally-encoded OXA carbapenemases and in *P. aeruginosa* by hyperexpression of efflux pumps, cell wall impermeability, or hyperproduction of AmpC beta-lactamases (17). While carbapenem resistance is readily detected *in vitro* in *P. aeruginosa* and *A. baumannii*, detection of carbapenem resistance in Enterobacterales, e.g. *K. pnemoniae* and *E. coli*, is more challenging and required revision of minimum inhibitory concentration (MIC) breakpoints by The European Committee on Antimicrobial Susceptibility Testing (EUCAST) (18) and Clinical and Laboratory Standards Institute (CLSI) (19). Although carbapenem resistance in Enterobacterales is mostly mediated by carbapenemase production, from an infection control standpoint, it is very important to distinguish between CP and non-CP Enterobacterales as the genes encoding carbapenemases are generally located on mobile genetic elements (i.e., plasmids, transposons, and insertion sequences) and are easily transmissible to other Gram-negative organisms (20). On the contrary, carbapenem resistance in non-CP isolates commonly occurs due to the hyperproduction of ESBL or AmpC enzymes combined with a reduced cell wall permeability which is associated with a loss of organism fitness and reduced transmissibility (20). Detecting carbapenemase production and defining the carbapenemase type is crucial in monitoring the spread of successful epidemic clones, particularly of *K. pneumoniae*.

Classification of carbapenemases

Due to the large number and high diversity of β-lactamases, their classification is not an easy task (21). Classification schemes brought in a few decades ago are constantly updated and adjusted to the ever-growing number of emerging enzymes. In 1989, Karen Bush et al. presented a classification according to the functional characteristics of β-lactamases (22), while in 1980 Ambler proposed a classification by which β-lactamases can be distributed into four classes (A, B, C, and D) based on primary amino acid sequence homology (21). β-lactamases produced by Enterobacterales, which have a significant clinical role in compromising carbapenems and being epidemiologically most relevant, belong to the Ambler classes A, B and D (2, 4). *Enterobacter cloacae*, strain NOR-1, which produces carbapenemase from the Ambler class A, was the first CPE, isolated in 1993 (4).

Class A carbapenemases

Class A β-lactamases are susceptible in a varying degree to β-lactamase inhibitors such as clavulanic acid. They are often encoded on plasmids that can move by conjugation and, as a result, these enzymes are widespread sources of resistance. Broad-spectrum-β-lactamases and their variants ESBLs are examples of class A β-lactamases. The most significant representatives of the carbapenemases in the Ambler class A are *Klebsiella pneumoniae* carbapenemase (KPC), Guiana Extended-Spectrum beta-lactamase enzymes (GES), *Serratia marcescens* enzyme (SME-1), imipenem-hydrolyzing beta-lactamase enzymes (IMI), the non-metallo-carbapenemase-A enzyme (NMC-A), of which KPC is the most clinically and epidemiologically important (2, 4, 11). As new treatment options for class A carbapenemases (e.g. KPC), some combinations of β-lactam antibiotics and new β-lactamase inhibitors have recently been approved on the market. Avibactam is a first-in-class, non-β-lactam, β-lactamase inhibitor with a broad spectrum of activity, including activity against KPC enzyme (23). A combination of the third generation cephalosporin, ceftazidime and aviabactam was approved in the USA in 2015 and in Europe in 2016 (23). Meropenem/vaborbactam was approved by the US Food and Drug Administration (FDA) in 2017 as the first carbapenem β-lactamase inhibitor combination and the same combination got approval a year later in Europe (24). Imipenemcilastatin/relebactam was approved by FDA for use in the USA in 2019.

To date, more than 50 variants of KPCs are known (25), KPC-2 and KPC-3 being the most common (26). The KPCs show hydrolytic activity to all penicillins, cephalosporins, aztreonam and carbapenems (27). Genes bla_{KPC} are often not the only ones located on plasmid, but are frequently associated with genes responsible for resistance to other classes of antimicrobial agents such as quinolones, aminoglycosides, tetracyclines, trimethoprim and sulphonamides what makes KPC isolates multidrug- or even worse pandrug-resistant (26). Although *K. pneumoniae* is generally the most common bacterial species that produces this enzyme, KPC can also be found in some other species such as *E. coli, C. freundii, S. marcescens*, *Enterobacter* spp., and *Pseudomonas* spp. (26). The first isolate of *K. pneumoniae* producing KPC (KPC-1) was detected in the USA in a North Carolina hospital in 1996 and it was uncommon until 2001 when it spread to other states on the east coast of the USA (New York and New Jersey) and after that throughout the country (2, 27, 28). This carbapenemase spread not only across the USA but also worldwide because of its epidemic potential and clonality of *K. pneumoniae,* predominantly the sequence type (ST) 258 (12, 29). It is interesting how a small isolated USA territory, the island Porto Rico became a place of origin for some KPC variants that soon became endemic (27). The first *K. pneumoniae* KPC (KPC-2) was identified in 2005, in Paris, from urine and blood culture of a patient who had previously been treated in a New York City hospital, thus resulting from an intercontinental transfer (27, 30). The other reported cases across the USA were from Colombia in 2006 and from China and Israel a year later (31). Just as in France, the first KPC case in Israel was imported from the USA, leading to an increasing incidence of KPC producers during 2006, which quickly resulted in the emergence of a nationwide outbreak and an endemic site for KPCs (27, 32). However, the compliance with the infection control measures and guidelines has successfully stabilized the spread of these resistant strains (27, 32). *K. pneumoniae* clone ST258 predominates in many parts of the world but there are other successful clones like the clone ST11 which is responsible for the spread of KPC in China (27). The first Italian report on *K. pneumoniae* KPC dated from 2008, which soon evolved into an endemic situation (27). One of the first European countries to become endemic for *K. pneumoniae* KPC was Greece and the recent surveillance report from ECDC shows that Greece struggles with more than 60% of carbapenem-resistant isolates among invasive *K. pneumoniae*, which shows the highest prevalence in Europe (33, 34). A nationwide multicentric study demonstrated that KPC is the most common carbapenemase (66,5%) in Greece (35).

To sum it up, the worldwide spread of KPC producing *K. pneumoniae* is linked to the dissemination of a few very successful clones and has led to the endemicity areas in the USA, South America, Greece, Italy, China and Israel (29, 33).

Class B carbapenemases

The Ambler class B β-lactamases are zinc metalloenzymes commonly referred to as metallo-β-lactamases (MBLs). These enzymes degrade all

β-lactams except aztreonam (4, 36). Since zinc ion(s) is (are) required for the activity of these enzymes, it follows that ethylene-diaminetetraacetic acid (EDTA) and other metal cation chelators are inhibitors of these carbapenemases (37). Other β-lactamase inhibitors, including a novel non-β-lactam, β-lactamase inhibitor avibactam are not effective against MBLs (23). The Verona integron–encoded metallo-β-lactamase (VIM), imi-penemase (IMP) and the New Delhi metallo-β-lactamase-1 (NDM-1) are the most frequent β-lactamases that belong to this class of carbapenemases. MBLs are classified in three subclasses B1, B2 and B3 with clinically most relevant class B carbapenemases VIM, IMP and NDM belonging to the subclass B1 (37).

The VIM-producing Enterobacterales are mostly found in Europe, predominantly southern Europe and in the Mediterranean area (38). The first VIM-type enzyme was described in Italy in 1997, detected in an isolate of *P. aeruginosa* (37, 39). Currently, there are known more than 60 variants of VIM enzyme (25). The VIM producers have expanded around the world mostly because of *P. aeruginosa* isolates (38). This enzyme is not only detected in *P. aeruginosa*, but also increasingly in Enterobacterales, especially in *E. coli, Enterobacter* spp. and *K. pneumonia*, the latter being the most predominant one (36). The first VIM-producing *K. pneumoniae* in Greece was detected in 2002 in Athens and it disseminated rapidly over the country resulting in an endemic for MBL (33, 40). Apart from being endemic in Greece, VIM enzyme is also widely disseminated in Spain, Italy and Hungary where inter-regional spread was observed in 2014-2015 (33, 36).

A novel MBL gene, designated $bla_{\text{NDM-1}}$, encoding for New Delhi Metallo-β-Lactamase was reported for the first time in 2008 in *K. pneumoniae* and *E. coli* isolated from a Swedish patient of Indian origin after being hospitalized in New Delhi (38, 41). Among β–lactamases in class B, NDM is one of the most clinically significant. More than 20 variants of NDM have been assigned so far (25). According to amino acid identity, NDM-1 is not similar to other MBLs (38). Most NDM positive strains were reported from Asia, particularly in China and India (42). The Indian subcontinent (Pakistan, India, Sri Lanka, Bangladesh) is considered as an endemic and crucial reservoir of NDM producers (12, 38). In India, NDM-1 is the most observed carbapenemase (43). Other likely-reservoirs accountable for the spread of NDM producers are considered to be the Balkans, the Arabian Peninsula and North African countries (38). Due to its close relationship with India and Pakistan, the UK consequently has a significant incidence of *K. pneumoniae* NDM isolates (38, 43). In Europe, an inter-regional spread of NDM producers was noted in three European countries: Poland, Romania and Denmark in 2014-2015 (33).

The first report of IMP-1 detected in *P. aeruginosa* was published in 1991 in Japan (44). These carbapenemases are not common in Enterobacteriales and such isolates are mostly detected in the South Pacific and Asia (e.g. Japan, Taiwan, eastern China) (38, 45), and are rare in Europe (33). So far, more than 80 variants of IMP have been assigned (25).

Class D carbapenemases

The Ambler Class D β-lactamases mainly consist of oxacillinases (OXAs) and so far there are more than 800 variants with different hydrolytic spectrum (25). Some of them have a narrow-spectrum β-lactamase activity, the others are ESBLs, and some of them act as carbapenemases (26). The latter can be found in specialized literature also as the carbapenem-hydrolyzing class D β-lactamases (CHDLs) (46). The CHDLs weakly hydrolyze carbapenems, and if not associated with other resistance mechanisms (such as altered permeability), do not pose a threat for developing high resistance to carbapenems (38). Furthermore, extended-spectrum cephalosporins are mostly not their substrate, but they hydrolyze temocillin (26, 38). The clavulanic acid, tazobactam and sulbactam, as classical β-lactamase inhibitors, are not successful against enzymes in this class (46). However, a novel β-lactamase inhibitor avibactam, which has already been mentioned above, exhibits both an activity against enzymes in the Ambler class A and class C but also inhibits certain enzymes in class D (eg. OXA-10, OXA-48) (23).

The OXA carbapenemases can be subcategorised in two groups, one is associated with A. baumannii, and the other one, the OXA-48-like carbapenem-hydrolysing oxacillinases are products of *Enterobacterales* and include several variants of which the most important are the following: OXA-48, OXA-162, OXA-181, OXA-204, OXA-232, OXA 244, OXA 245, OXA-247, OXA-436, OXA-484, and OXA-519 (47). The enzyme OXA-48

emerged in Turkey in 2001 where this carbapenemase was described in an isolate of *K. pneumoniae* (2, 48). After a rapid expansion throughout Turkey and causing nosocomial outbreaks, OXA-48 enzyme spread to the Middle East, North Africa and Europe (49). The gene $bla_{\text{OXA-48}}$ is located on the plasmid that does not possess any other genes for resistance, however due to its high conjugation rate, it easily and rapidly spreads among other species of order Enterobacterales (38). Besides the Mediterranean area, OXA-48 producers have been reported in some other European countries (26). In contrast to Europe, the OXA-181 enzyme variant identified in India is the dominant carbapenemase from the OXA-48-like subfamily, being spread from there to other countries (38). Although OXA enzymes can be found worldwide, there are areas such as countries of North and South America, Australia, Russia and China that have recorded low prevalence of OXA-48 produ-cers (38, 43). An inter-regional spreading is characteristic for Europe, Belgium, Spain, Romania and France, whereas Malta and Turkey are endemic areas for OXA-48 carbapenemase as assessed in 2014-2015 (33). Along with these two countries in Europe, some countries of North Africa (e.g. Morocco, Libya, Egypt) as well as India are also considered endemic areas for *K. pneumoniae* OXA-48 (43). Unlike KPC and VIM producers, OXA-48 producing Enterobacterales are still rare in Greece (33).

Epidemiology of cre in Europe

High rates of carbapenem resistance in invasive *K. pneumoniae* are seen in some countries that regularly report to EARS-Net and CAESAR surveillance networks (34, 50). Greece and Belarus reported proportions more than 50%, and Georgia, Italy, Romania, the Russian Federation, Serbia, Turkey and Ukraine reported proportions between 25% and 50%. However, resistance rates do not necessarily reflect the true epidemiological burden of resistant strains. Therefore, in 2012, the ECDC initiated the „European survey of carbapenemase-producing *Enterobacteriaceae* (EuSCAPE)“ project to collect data about occurrence of CPE across Europe and improve laboratory capacity for diagnosis and surveillance of CPE in European countries (33). The participating countries were the 28 EU Member States, Iceland, Norway, the seven EU enlargement countries (Albania, Bosnia and Herzegovina, Kosovo, Montenegro, the former Yugoslav Republic of Macedonia, Serbia and Turkey) and Israel, in total 38 countries (33). In 2013, a national expert of each of the EuSCAPE participating countries got a questionnaire on CPE issue and health system responses, and the second one followed in 2015, after finishing the project. A novel epidemiological staging system was applied to describe the magnitude of CPE and while comparing the two questionnaire reports, it was proven that the epidemiological situation obviously worsened during a 2-year period (2013-2015). A rapid spread of OXA-48- and NDM-producing Enterobacterales was reported. In 2017, the European Antimicrobial Resistance Gene Surveillance Network (EURGen-Net) was set up to carry out the surveys of carbapenem and/or colistin-resistant Enterobacterales in Europe as a continuation of the EuSCAPE project (5). This time all 37 participating countries had confirmed CPE isolates (Israel did not participate). In most countries the epidemiological situation didn’t change, however it worsened in 11 countries, which however showed a favorable outcome after the implementation of control measures in Slovenia (5). The overall highest incidence of CP *K. pneumoniae* is found in southern and southeastern Europe with Greece, Italy, Malta and Turkey mostly affected with endemic situation for CPEs (5).

CONCLUSIONS

CPEs present a serious threat to public health and limit treatment options for critically ill patients. It is of utmost importance, therefore, to develop laboratory capacity and ability to detect carbapenemase production in Enterobacterales as this has important infection control implications and is essential in organizing a reliable surveillance network. Based on local, national and international surveillance data a set of measures tackling prudent antimicrobial prescribing and infection prevention and control precautions should be implemented. Local and national clinical guidelines for antimicrobial use should be developed based on local epidemiology of MDROs. When developing guidelines, special care should be taken to preserve the efficacy of broad-spectrum antibiotics such as carbapenems through promoting bacteriological testing and targeted narrow spectrum antibiotic therapy. Whenever possible de-escalation policy should be implemented. To increase bacteriological testing and enable targeted antibiotic

therapy, clinical involvement of microbiologists and laboratory availability seven days a week should be encouraged. Communication of microbiologists, infectious disease specialists, infection control practitioners among each other and with other health care workers on daily basis is crucial in tailoring antimicrobial therapy and restricting the spread of resistant clones. Among MDRO, CP *K. pneumoniae* presents a special challenge for the laboratory, clinicians and epidemiologists as carbapenem resistance in Enterobacterales is not as readily detected as in other bacterial species, there are very few options for treatment left and it seems that the burden of carbapenem-resistant *K. pneumoniae* is increasing at the accelerating speed in Europe.

CONFLICT OF INTERESTS

The authors declare no conflict of financial or non-financial interests.

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Date of receipt of the manuscript: 18/11/2020
Date of acceptance for publication: 19/12/2020

OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

RESEARCH ARTICLES – ARTICOLE DE CERCETARE – ARTICLES DE RECHERCHE – НАУЧНЫЕ СТАТЬИ

BIOLOGICAL SCIENCES

OPEN ACCESS

DIETARY IMPROVEMENT OF THE IRON STATUTE OF THE RATS WITH EXPERIMENTAL ANEMIA

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DOI: 10.38045/ohrm.2021.1.02 UDC: 616.155.194.8-092.9+613.2

Key words: *iron anemia, rats, in vivo research, fortified bread, lactic acid fermentation, peripheral blood parameters, serum iron.*

Introduction. *One of the major problems characterizing the nutritional status is the iron deficiency, which leads to decreased immunity, increased incidence of infectious diseases and anemia. Obviously, only well-thought-out corrections can solve these disorders.*
Material and methods. *In order to know the influence of the method of manufacture for iron-fortified products on the iron level, a series of in vivo research was carried out, on a batch of 21 white rats, Wistar line, which were induced experimental drug anemia (EDA). The blood analysis was performed in three stages: initially, after the induction of EDA and after the completion of the experiment.*
Results. *Investigations showed out that during sufficient iron intake rehabilitation of the body iron reserve depends considerably on the presence of antinutritive factors. In order to decrease the influence of these factors, the production method used for fortified products with mineral origin micronutrients (iron) could be based on the use of the lactic-acid fermentation procedures using wheat bran.*
Conclusions. *The research carried out on laboratory animals with induced experimental anemia showed that, in the case of the group fed with bread manufactured by the acid-lactic fermentation method, the concentration of serum, iron was completely restored during the experiment, and in the rats fed with fortified bread prepared by the traditional method, the serum iron concentration was only partially restored, 40-45%.*

Cuvinte cheie: *anemie feriprivă, şobolani, cercetări in vivo, pâine fortificată, fermentaţie acido-lactică, parametri ai sângelui periferic, fier seric.*

AMELIORAREA DIETARĂ A STATUTULUI DE FIER LA ŞOBOLANI CU ANEMIE EXPERIMENTALĂ
Introducere. *Una dintre problemele majore care afectează statutul nutriţional este insuficienţa de fier, care duce la scăderea imunităţii, la sporirea incidenţei maladiilor infecţioase şi a anemiilor. Şi, evident, numai corecţiile echilibrate pot remedia aceste dereglări.*
Material si metode. *În scopul de a elucida influenţa metodei de fabricare a produselor fortificate cu fier asupra aportului acestuia în organism, au fost efectuate o serie de cercetări in vivo, pe un lot de 21 şobolani albi, linia Wistar, cărora li s-a indus anemie experimentală pe cale medicamentoasă (AEM). Analiza sângelui a fost efectuată în trei reprize: iniţial, după inducerea AEM şi, apoi după finalizarea experimentului.*
Rezultate. *Investigaţiile au arătat, că chiar şi la un aport suficient de fier, reabilitarea rezervei de fier a organismului depinde considerabil de prezenţa factorilor antinutritivi. Pentru a reduce influenţa acestor factori, metoda de fabricaţie aplicată, pentru produsele fortificate cu micronutrienţi de origine minerală (fier), s-ar putea baza pe utilizarea procedurilor de fermentare acido-lactică, folosind tărâţe de grâu.*
Concluzii. *Cercetările efectuate pe animale de laborator, cu anemie experimentală indusă, au demonstrat, că în cazul grupei hranite cu pâine fabricată prin metoda cu fermentaţie acido-lactică, concentraţia fierului seric s-a restabilit complet pe durata experimentului, iar la şobolanii alimentaţi cu pâine fortificată, preparată prin metoda tradiţională, concentraţia fierului seric s-a restabilit doar parţial, în proporţie de 40-45%.*

ABBREVIATIONS: ***AA***, after anemia was induced; **ASD**, after special diet administration; ***BA***, before anemia was induced; ***eALAS***, erythroid-specific delta-aminolevulinic acid synthase; ***IFB***, iron-fortified bread; ***HB***, hemoglobin concentration; ***HT***, hematocrit; ***IRE***, iron-responsive element; ***IRE-BP***, iron-responsive element-binding protein; LYM%, percent report between lymphocyte and the total lymphocytes number; ***LYM***, the absolute number of lymphocytes; ***MC***, myelocytes; ***MCHC***, average body concentration of the hemoglobin; ***MCV***, the average volume of erythrocyte; ***mMC***, metamyelocytes; ***MPV***, the average value of the platelet; ***mRNA***, messenger ribonucleic acid; ***PA***, phytic acid; ***PDW***, platelet distribution amplitude; ***PHZ***, phenylhydrazine; ***P-LCP***, report between big platelet and the total platelet values; ***PLT***, platelet number; ***RBC***, erythrocyte number; ***RDW***, distribution amplitude of the erythrocytes; ***SI***, serum iron; ***UNICEF***, United Nations International Children's Emergency Fund; ***WBC***, leukocyte number.

INTRODUCTION

Reduction of anemia is an important constituent of human health and a global nutrition target for 2025 (1). In low-income countries, the presence of anemia remains high and is a priority area (2). Up to half of the children and childbearing age women in developing countries and about 10% in developed countries are estimated to be iron deficient. Iron deficiency leads to impaired physical work performance, cognitive impairment, and adverse pregnancy outcomes. More and more evidences show that iron deficiency anemia in infants and toddlers can lead to irreversible development delays (3).

The study of the food consumption and nutritional status of Moldovan people, made with the support of UNICEF showed that the products used in women nutrition aged 18-45 years old contained only 28-53% of the iron daily amount (4). The consequence of this deficiency is the prevalence of iron deficiency anemia in 29% of the children aged 6-59 months (blood hemoglobin concentration <110 g/L) and in 26% of non-pregnant women of reproductive age (15-49 years) (1). The level of significance of anaemia for public health is considered to be moderate.

Three intervention strategies are available to prevent iron deficiency and, therefore, iron deficiency anemia (5). These are supplements, dietary diversification, and both targeted and untargeted food fortifications. Food fortification programs are cost effective methods of reducing the prevalence of iron deficiency (6). The effectiveness of a food fortification program depends on the consistent and uniform addition of iron compounds to appropriate foods, such as flour, which are widely consumed by the target population (7, 8). Mineral intake and bioavailability are critical factors for meeting mineral nutritional needs (9, 10). Whole grain bread contains high levels of potassium, magnesium, iron, and zinc, but the presence of phytic acid compromises mineral and trace element absorption in humans (11). During bread making the content of phytic acid decreases due to the action of phytases in the dough (12). Nevertheless, if very little phytate is hydrolyzed in unleavened whole meal breads including breads containing sodium bicarbonate, phytic acid hydrolysis occurs during all stages of yeast bread making. Reduction of phytic acid content in different bread types varies between 13% and 100%, with the lowest decrease being in unleavened breads. The substantial decrease of phytic acid in whole wheat products can improve mineral availability in humans. Because consumption of whole grain breads is increasing, a whole wheat bread with low phytic acid levels and increased mineral bioavailability would be beneficial and attractive in improving mineral status and, hence, preventive nutrition.

A certain number of investigations showed that lactic-acid bacteria is capable to degrade phytic acid, and the lactic-acid fermentation increases calcium, magnesium, and iron solubility during *in vitro* investigations (13). Taken together, phytic acid degradation and lactic acid forming improved minerals bioavailability in the bread prepared through the lactic-acid method.

Most of the recent technological innovations, which decrease the duration of the transformation period of the agricultural material, can lead to a decrease of biological value of the foodstuffs and of its quality (14). But traditional diets of each region include different transformation procedures of the primary agricultural material, which are characteristic for the particular region and lead to the production of foodstuff that have physico-chemical and flavor characteristics, accepted by the majority of consumers. There is only one possibility to reduce the impact of the antinutritional factors on the foodstuff processing – development of technologies based on traditional procedures, verified over centuries, which

allows to valorize the reserves of each particular region (15, 16, 17). As bread represents the most essential foodstuff in the human nutrition, it is obvious that the main tendency is led towards development of such bread making procedures that would ensure a maximum biological value of the product (18, 19).

It was previously established that no-heme iron from the bread prepared through the lactic-acid method shows a higher bioavailability *in vitro* than the bread prepared through the traditional method (20). Simultaneously, a decrease of the phytic acid during *in vitro* gastro-intestinal digestion was identified. This shows that there is a direct relationship between potential bioavailability of iron in bread products and the hydrolysis degree of the phytates. *In vivo* studies, performed on rats showed that peripheral blood parameters of the laboratory animals are significantly improved compared to the control group, these were fed with fortified bread. But investigations did not allow to establish the influence of bread making procedure on the iron bioavailability because collected blood from both rat groups that were fed with a special diet (bread prepared through the traditional and lactic-acid fermentation method) do not show any distinctive differences.

In order to investigate the influence of bread making procedure used for production of iron fortified products on the body iron status a range of *in vivo* investigations were drown on laboratory animals in which anemia was experimentally induced.

MATERIAL AND METHODS

Materials

For the investigations white wheat flour bread of high quality of autochthon production was used (21). As additive iron sulfate (II) was used ($FeSO_4 \cdot 7H_20$) accepted as food additive by "Codex Alimentarius".

Bread making procedures

Bread was prepared by indirect (bi-phase) method, additive being added at the final knitting stage and through lactic-acid method. In order to reproduce the production of bread from integral wheat flour, reconstituted integral flour was used (flour with bran). For the production of bread with yeast 6 kg integral wheat flour (4.65 kg wheat flour plus 1.35 kg bran) was mixed with 3.6 L distilled water and 150 g yeast (*Saccharomyces cerevisiae*). For lactic-acid fermentation the dough was prepared through the mixing of 1.2 kg integral wheat flour (0.93 kg wheat flour and 0.27 kg bran) with 600 mL distilled water. After one day of natural fermentation at 30°C, the dough was mixed with 4.8 kg, integral wheat flour and 3 L distilled water. Bread was baked, dried, milled and introduced in the diet of rats.

In vivo procedures

Experiments were performed on a group of 21 white rats Wistar strain with the body weight 160-180 g which divided into 3 groups (7 rats each) in which experimental anemia was induced. Experimental anemia was reproduced for the laboratory female rats through repeated intramuscular injection of a 2% solution of phenylhydrazine (PHZ) prepared on physiological solution for 2 weeks (6 injections). Afterwards, the animals were kept on a special diet for 21 days, which included nutritional supplement bread, prepared via various methods and containing certain iron amount.

The first control group (Ia) represented animals, to whom were administered bread prepared via the classical biphase method without iron as nutritional supplement. The second group (IIa) included animals, which were fed with bread prepared by traditional bi-phase method with an addition of 8 mg iron/100 g reconstituted flour. The third group (IIIa) included animals, which were fed with bread prepared by lactic-acid fermentation method with addition of 8 mg iron/100 g reconstituted flour.

Analyses of blood samples

Biological material – peripheral blood collected from the laboratory animals was sampled before PHZ-induced anemia (BA), after PHZ-induced anemia (AA) and after special dietary administration (ASD) (22). Blood was collected in 1.5 mL Eppendorf test tubes. Blood indexes were determined in the peripheral heparinized blood in the hematology analyzer PCE-210 (ERMA, Japan). The following parameters were determined: red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW), platelets (PLT), mean platelet volume (MPV), platelet distribution width (PDW), platelet larger cell ratio (P-LCR), white blood cell count (WBC), absolute number of lymphocytes (LYM) and percentage of lymphocytes (LYM %), mono-

cytes (MONO), granulocytes (GRAN), unsegmented and segmented neutrophils, eosinophils, normocyte, serum iron (SI).

Blood serum was obtained through centrifugation of the peripheral blood at 2500±500 min^{-1} for 15 minutes. After centrifugation, blood serum was transferred in clean Eppendorf test tubes for single use and kept in the refrigerator at 4±1°C till the end of the experiments. The determination of the serum iron was performed by cromazurole test (Elitech, France) according to the annexed analyze instructions.

The research was approved by the Research Ethic Board of the *Nicolae Testemiteanu* State University of Medicine and Pharmacy of the Republic of Moldova, (favorable opinion of June 19, 2011).

Statistics

The analysis of the results variety was carried out by least square method with application of Student coefficient and Microsoft Office Excel program version 2007 (Microsoft Excel® 2007, Microsoft Corp., Redmond, WA). The differences were considered statistically significant if probability was greater than 95% (p-value <0,05). All assays were performed at room temperature 20±1°C. The experimental results are expressed as average ±SD (standard deviation).

RESULTS

Blood parameters analysis collected from the animals with PHZ-induced anemia are presented in table 1.

Leukocyte formula for the laboratory animals before PHZ-induced anemia (BA), after PHZ-induced anemia (AA) and after special dietary administration (ASD) is presented in table 2.

Table 1. Blood parameters analysis collected from animals before the PHZ-induced anemia (BA), after PHZ-induced anemia (AA) and after special dietary administration (ASD).

Blood parameters	Ia			IIa			IIIa		
	BA	AA	ASD	BA	AA	ASD	BA	AA	ASD
RBC (10^{12}/L)	6.47± 0.05	2.16± 0.05	6.55± 0.05	6.31± 0.05	2.96± 0.05	6.65± 0.05	6.10± 0.05	2.64± 0.05	6.72± 0.05
HGB (g/L)	136±1	59±1	131±1	139±1	82±1	147±1	133±1	73±1	142±1
HCT (L/L)	0.409± 0.02	0.229± 0.02	0.395± 0.02	0.400± 0.02	0.299± 0.02	0.41± 0.02	0.391± 0.02	0.279± 0.02	0.401± 0.02
MCV (fL)	63.6± 0.1	113.9± 0.3	60.4± 0.2	63.1± 0.1	101.7± 0.3	63.3± 0.2	64.4± 0.1	106.5± 0.3	62.7± 0.2
MCHC (g/L)	334.5± 0.5	275± 1	330.5± 0.6	350.0± 0.5	278± 1	361.5± 0.6	339.0± 0.5	261± 1	354.0± 0.6
RDW (fL)	77.4± 0.7	125.9± 0.6	78.8± 0.6	71.7± 0.7	108.0± 0.6	74.9± 0.6	83.2± 0.7	113.0± 0.6	74.8± 0.6
PLT (10^9/L)	523±3	184±3	682±5	345±3	705±6	633±5	539±3	485±5	624±5
MPV (fL)	15.9± 0.1	16.3± 0.2	14.5± 0.1	15.2± 0.2	14.0± 0.2	13.9± 0.1	14.8± 0.2	14.8± 0.2	14.0± 0.1
PDW (fL)	21.7± 0.3	16.2± 0.3	18.2± 0.2	23.3± 0.3	14.8± 0.3	16.0± 0.2	16.6± 0.3	15.2± 0.3	16.4± 0.2
P-LCR	0.353± 0.003	0.343± 0.005	0.217± 0.005	0.346± 0.002	0.166± 0.007	0.155± 0.005	0.264± 0.003	0.242± 0.002	0.158± 0.005
WBC (10^9/L)	11.3± 0.3	5.3± 0.3	9.9± 0.3	9.6± 0.3	9.0± 0.3	9.7± 0.3	11.2± 0.3	8.5± 0.3	9.8± 0.3
LYM (10^9/L)	6.4±0.1	4.4±0.1	6.0±0.1	5.4±0.1	7.3±0.1	4.7±0.1	6.7±0.1	7.2±0.1	5.7±0.1
SI (µmol/L)	n.d.	n.d.	10.87± 0.87	n.d.	n.d.	18.37± 1.63	n.d.	n.d.	41.1± 1.1

RBC decreased significantly more than 2 times, after inducing anemia in all experimental groups. The control group (Ia), considered as the reference one, revealed the normal levels of RBC, which reverted due to special diet administration.

As for the IIa and IIIa groups fed with fortified bread (FB), an improvement of this index compared to the initial parameters before the anemia was induced when the RBC increased with 5% and 10% respectively.

After PHZ-induced anemia, the HGB decreased almost twice in all 3 investigated groups. Special food administrated to the animals from the Ia group leaded to rehabilitation of the HGB amount up to normal levels. In the case of the animals fed with FB (IIa and IIIa) an increase of the HGB was noticed, the values were more than 7% higher compared to the indexes before anemia. This fact proved the curative effect of the iron FB, due to its significant bioavailability.

Table 2. Leukocyte formula for the laboratory animals before PHZ-induced anemia (BA), after PHZ-induced anemia (AA) and after special dietary administration (ASD).

Blood parameters	Ia			IIa			IIIa		
	BA	AA	ASD	BA	AA	ASD	BA	AA	ASD
MC and mMC (%)	0	0	1.8± 0.57	0	1.2± 0.9	3.4± 0.49	0	0	4.5± 0.51
Unsegmented neutrophils (%)	1.7± 0.20	0.4± 0.20	0.8± 0.20	1.0± 0.70	0.8± 0.20	1.0± 0.20	0.7± 0.30	0.4± 0.20	1.0± 0.3
Segmented neutrophils (%)	29.0 ± 2.0	40.0± 7.0	34.0± 2.0	28.0± 2.0	32.0± 7.0	31.0± 3.0	31.0± 3.0	30.0± 2.0	26± 2.0
Eosinophils (%)	0.7± 0.3	0.4± 0.2	0.6± 0.3	0.8± 0.5	1.2± 0.6	0.8± 0.3	0.8± 0.5	0.4± 0.2	0.8± 0.3
Basophils (%)	1.0± 0.4	0.4± 0.2	0.8± 0.2	1.2± 0.9	0.8± 0.2	1.0± 0.7	1.0± 0.4	0.2± 0.4	1.0± 0.3
LYM (%)	60.0± 2.0	51.0± 6.0	52.0± 1.7	62.0± 1.0	56.0± 5.0	52.0± 2.5	59.0± 3.0	61.0± 3.0	55.0± 1.4
MONO (%)	7.6± 1.1	7.6± 1.1	10.0± 2.0	7.0± 1.2	8.6± 1.7	10.0± 1.8	7.0± 0.8	8.0± 0.5	11.8± 1.8
Normocyte	-	++++	-	-	++	-	-	-	-

The HCT decreased significantly after PHZ-induced anemia with approximately 25-44%, then rehabilitated reaching the initial level in the control group and even insignificantly exceeded the initial value for the animal groups fed with FB.

The MCV significantly increased after the PHZ-induced anemia with up to 60-79%, which indicated the presence of a severe anemia and can be considered as an accommodation and compensation reaction. The aforementioned parameter reverted to its normal values after 3 weeks of fortified food administration (groups IIa and IIIa). The value of the final parameter reached the initial level in the control group.

The MCHC has been reduced with about 120-150 units (with 18-21%) after the PHZ-induced anemia, which did not revert to the initial value in control group. For the IIa and IIIa groups of animals, which were fed with FB, there was slight increase tendency for the MCHC compared to the initial values.

The RDW increased considerably (with 40-50 units, 35-63%) after the PHZ-induced anemia, then reverted to the normal values for the control group as well as for the groups fed with FB.

PLT varied considerably after the PHZ-induced anemia was reestablished due to a special diet administration for the control group, as well as for the animal groups fed with FB.

The MPV did not change significantly after the PHZ-induced anemia, however, after the special diet, the above mentioned parameter had a slight decrease tendency, which maintained at almost the same values for all the groups studied.

The PDW did not decrease essentially after the anemia was induced and reverted to the initial value after the special diet administration for the control group as well as for the iron FB fed groups.

The P-LCR decreased significantly, especially for the groups IIa and IIIa, where this parameter due to a special diet administration maintained at low values.

Once anemia was induced by phenylhydrazine the WBC decreased, especially for the control group – Ia (tab. 1). After the special diet administration during 3 weeks, the WBC reverted to the normal values for all groups (Ia – IIIa).

The LYM varied considerably after the PHZ-induced anemia and remained at low levels, being inferior to the ones registered before the anemia was induced in all the groups studied.

The table 1 shows the evolution of serum iron (SI) after application of special diet in the presence of PHZ-induced anemia. It was established that in the experimental groups with PHZ-induced anemia after the special diet administration the level of the SI increased from 10.87±3.67 µmol/L for group Ia to 41.10±17.23 µmol/L for group IIIa, where animals were fed with fortified bread with iron prepared by lactic-acid method (20).

Our data revealed that after PHZ-induced anemia, unsegmented neutrophils slightly decreased in the peripheral blood, while segmented tended to increase, as well as normocytes were formed (tab. 2).

After induction of anemia, the LYM% in the leukocyte formula did not undergo any relevant changes for all three groups of investigated animals. This parameter decreased slightly compared to the normal values in all studied groups after the administration of the special diet rich in iron. The decrease was followed by elevated percentage of monocytes and granulocytes in all studied groups, which slightly exceeded the initial values after the administration of the special diet.

Therefore, these modifications were more obvious for the animals fed with fortified bread prepared by lactic-acid fermentation.

DISCUSSIONS

The circulating red blood cells (erythrocytes), their precursors, and all body elements involved in the production of red blood cells make up the concept of erythron (23), and the state of the erythron can be determined by the number of red blood cells (RBC), HBG concentration, HCT level (volume of packed cells) and also on the basis of erythrocyte indices – MCV, MCHC, RDW.

The present study showed that the average level of erythron indices decreased after PHZ-induced anemia in all experimental groups and within the same limits as the average content of HGB in red blood cells – by 25-30%.

The results showed that erythrocyte indices (especially the MCHC index, which reflects the degree of erythrocyte saturation with hemoglobin) returned to the initial level after ASD, especially in group IIIa, where the animals were fed with iron-fortified bread obtained by lactic acid fermentation.

A decrease in MCHC (hypochromia) was caused by iron deficiency and a decrease in HBG synthesis occurred after modeling of PHZ-induced anemia. Thus, the administration of iron-fortified bread prepared by the method of lactic fermentation significantly improved the impaired erythron state caused by PHZ-induced anemia.

These data correspond to the results (24), which showed that foods fermented with lactic acid increase the absorption of Fe in human subjects, possibly by lowering the pH, activating phytases and forming soluble Fe complexes and organic acids.

SI concentration is an indicator of the iron quantity present in the plasma, fixed by a specific protein – transferrin. Based on the obtained data regarding iron absorption process, transport, hoarding, usage and elimination, the plasma iron balance could be established, as well as body iron requirements in different physiological environments.

In different disorders, the SI assessment indicates a decrease of the serum iron towards severe iron deficiency and an increase of this parameter for iron overdose, as well as megaloblastic anemia, hemolytic anemia and sideroblastic anemia. Commonly the iron transport parameters do not change, until body iron reserves are completely exhausted. The SI is the main indicator of iron therapy response during the convalescence period, following a severe anemia.

Regulation of the iron absorption is possible due to the intervention of two proteins from the intestinal mucous membrane (especially from duodenum), namely ferritin and a protein similar with serum transferrin named mucous transferrin (25). In iron deficiency, the concentration and synthesis speed increases in the mucous and the synthesis of the apoferritin from the duodenal cells decreases, this means that the absorbed iron will not be fixed in the cells as ferritin but will be transferred towards the plasma. On the other hand, in iron overcharge, the transferrin synthesis in mucous is reduced, however, the apoferritin production increases, which fixes the iron in the cells as in a non-absorbable form, being then released from the body through feces, when the cells that fixed them exfoliate.

Systemic iron homeostasis is regulated by the interaction of the peptide hormone, hepcidin and the iron exporter, ferroportin (26, 27), which coordinate the iron acquisition and use. Hepcidin levels are increased during iron sufficiency and inflammation and are decreased in hypoxia or erythropoiesis. Hepcidin is a negative regulator of iron export. Hepcidin binds to cell surface ferroportin, inducing ferroportin, degradation and decreasing cellular iron export.

Recently, the role of other protein structures, especially of the transferrin receptors and the delta aminolevulinic acid from the cells of the erythroid series (eALAS) has been demonstrated, this being the key enzyme in the heme porphyrin component synthesis pathway. It was also observed that protein synthesis and of the abovementioned enzymes are controlled by a "sensor" capable to feel the iron availability of the cells. This sensor is a 28-nucleotide messenger ribonucleic acid (mRNA) fragment. Due to a specific protein binding, the sensor modifies the mRNA translation process for ferritin, transferrin receptors and eALAS, which regulate the protein synthesis within the ribosome.

The abovementioned nucleotide fragment is called the iron responsive element (IRE) and the binding protein is called IRE-BP. It was proved that depending on the iron bioavailability in the cells, IRE-BP modifies its binding affinity towards IRE and affects the translation of the mRNA (28).

When the iron amount in the cells decreases, IRE-BP increases its affinity towards nucleotide fragment IRE of the messenger nucleic acids, which encodes ferritin and eALAS synthesis, restricts the translation of these messenger nucleic acids and reduces ferritin and eALAS synthesis. At the same time, the IRE-BP complex produces a stabilization of the mRNA, which encodes the receptors for the transferrin, leading to an increase of the synthesis of these receptors.

Conversely, an increase of the iron amount from the cells decreases the IRE-BP affinity towards nucleic fragment IRE from the specific messenger nucleic acids (mRNA) that encode the ferritin and eALAS, thus their translation is performed in optimal conditions and the protein and the aforementioned enzyme synthesis is amplified. Simultaneously, when IRE-BP cannot bind to IRE from the mARN, which encodes the receptors for transferrin, the mARN is less stable and the synthesis of aforementioned receptors decrease.

Thus, the intake of iron fortified bread, prepared by lactic-acid fermentation method leads to normal iron levels and better major blood indexes in phenylhydrazine – induced anemia. The bread enables to fulfill the serum iron reserves for the laboratory animals, since the lactic acid fermentation inhibits the activity of phytates, which are the antinutrients present in flour that commonly make iron unavailable for absorption.

CONCLUSIONS

1. During a severely induced anemia in laboratory animals, the rehabilitation of blood biochemical indices occurred similarly in rats from all groups, fed with iron-fortified bread, due to an increased iron intake.

2. In case of the group of rats, fed with bread prepared by lactic acid fermentation method, the serum iron amount was rehabilitated completely during the experiment (21 days).

3. For the experimental group, fed with fortified bread that was prepared by traditional method, the serum iron concentration rehabilitated only partially 40-45%, compared to the initial value (before the anemia was induced).

4. The control group rats, fed with non-fortified bread prepared via the traditional method, showed only 1/3 serum iron amount out of the normal range, after the experiment was finished.

5. The leukocyte count of the blood samples, collected from the animals with and without induced anemia, showed that the intake of iron-fortified bread, prepared via the lactic-acid fermentation method, leads to normal and even improved major blood indices, since it enables to better rehabilitate the serum iron levels within the laboratory animals.

6. The experimental investigations revealed that even in case of a sufficient iron intake, the rehabilitation of the body iron reserve depends considerably on the presence of the anti-nutritive factors (such as phytates in this case).

7. In order to decrease the impact of these factors, the manufacture of the bread products fortified with micronutrients of mineral origin (iron) could be based on the use of the procedures that involve lactic – acid fermentation, developed on wheat bran; development of these procedures can increase micro- and macronutrients bioavailability in the bread.

ACKNOWLEDGMENT

Authors would like to thank State Project 20.80009.5107.09 - *Improvement of food quality and safety by biotechnology and food engineering.*

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Date of receipt of the manuscript: 10/04/2020
Date of acceptance for publication: 25/09/2020

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OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

MEDICAL SCIENCES

OPEN ACCESS

EVALUAREA RISCULUI PENTRU STAREA DE SĂNĂTATE A POPULAȚIEI ÎN FUNCȚIE DE COMPOZIȚIA APEI POTABILE

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DOI: 10.38045/ohrm.2021.1.03 CZU: 613.9:628.1.033

Key words: *sanitary-chemical indicators of water, population morbidity, correlation, risk.*

HEALTH RISK ASSESSMENT OF THE POPULATION IN FUNCTION TO THE COMPOSITION OF DRINKING WATER

Introduction. *Drinking water includes a multitude of natural elements, with a strong connection between them, which through the dynamics of their modification in many cases influence the health of the population.*

Material and methods. *The quality of drinking water from the Prut river ecosystem was studied (water from aqueducts fed from the river, water from artesian wells, water from adjacent wells), health status of the population from riparian localities (Lipcani, Ungheni and Cahul). The correlations between these two indicators and the risk of population disease caused by water quality were calculated. Hygienic, epidemiological, sanitary-chemical, and statistical methods of investigation were used.*

Results. *Following the study, the existence of small and medium correlative dependencies between the water quality indices from the aqueduct fed from the Prut river and the prevalence of the main groups of nosology of morbidity was highlighted. High direct correlative dependencies of health indices on water quality in artesian wells and wells were quantified. The values of the risk of genito-urinary, osteoarticular, digestive system, blood diseases/anemia, etc. were determined.*

Conclusions. *Finally, there was a causal relationship between the factors investigated and the health of the population, highlighting the risk of developing nosological forms.*

Cuvinte cheie: *indicatori sanitaro-chimici ai apei, morbiditatea populației, corelație, risc.*

Introducere. *Apa potabilă conține o multitudine de elemente naturale, strâns legate între ele, care, prin dinamica modificării lor, deseori, influențează starea de sănătate a populației.*

Material și metode. *S-a studiat calitatea apei potabile din ecosistemul râului Prut (apa apeductelor alimentate din fluviu, apa apeductelor alimentate din sonde, apa din fântânile din adiacență), starea de sănătate a populației din localitățile riverane (Lipcani, Ungheni și Cahul). S-au calculat corelațiile între acești doi indicatori și riscul de îmbolnăvire a populației, cauzat de calitatea apei. S-au folosit metode de investigare igienice, epidemiologice, sanitaro-chimice, statistice.*

Rezultate. *Studiului efectuat a evidențiat existența dependențelor corelative mici și medii între indicii calității apei din apeductul alimentat din r. Prut și incidența sporită a principalelor grupe de nosologii ale morbidității. S-au cuantificat dependențe corelative directe mari ale indicilor de sănătate de calitatea apei din sondele arteziene și din fântâni. S-au relevat valorile riscului de apariție a bolilor aparatului genito-urinar, ale celui osteoarticular, ale sistemului digestiv, a maladiilor sângelui (anemii) etc.*

Concluzii. *În final, s-a determinat o relație cauzală dintre factorii cercetați și starea de sănătate a populației, cu prezența riscului de dezvoltare a unor forme nosologice.*

INTRODUCERE

Reprezentând o necesitate vitală pentru organism (1, 2, 3), apa poate produce atât efecte directe (prin calităţile sale biologice, chimice, fizice) (4, 5), cât şi indirecte (prin sol, produse alimentare) (6) asupra sănătăţii populaţiei.

Luând în considerare fenomenele de cauză – efect dintre calitatea apei potabile şi starea de sănătate a populaţiei, devine posibilă argumentarea şi elaborarea măsurilor de prevenţie, îndeosebi bazate pe particularităţile corelative ale indicatorilor şi pe datele estimării riscului de îmbolnăvire (7).

MATERIAL ŞI METODE

Ca obiect de studiu, au servit sursele de aprovizionare cu apă potabilă din ecosistemul râului Prut (apa apeductelor alimentate din fluviu, apa apeductelor alimentate din sonde, apa din fântânile din adiacenţă) şi starea de sănătate a populaţiei din localităţile riverane (Lipcani, Ungheni şi Cahul).

Pentru a aprecia indicii stării de sănătate, s-a studiat morbiditatea retrospectivă (2005-2017) după adresabilitate a populaţiei din zonele selectate (formular 12) şi din localităţile cercetate. De asemenea, s-a examinat fişele medicale persona-le. Pentru a determina calitatea apei folosite în scop potabil, s-au efectuat investigaţii sanitaro-chimice sezoniere de laborator şi s-au analizat datele retrospective ale rezultatelor cercetărilor de laborator din Centrele de sănătate publică teritoriale, pentru perioada anilor 2005-2017. În scopul prelucrării statistice a materialului obţinut, s-a stabilit, prin tehnici speciale de evaluare computerizată, gradul de relaţionare între parametrii de sănătate şi caracteristicile apei potabile. S-au determinat valorile riscului de apariţie a bolilor aparatului genito-urinar, ale celui osteoarticular, ale sistemului digestiv, a maladiilor sângelui (anemii) etc.

REZULTATE

În urma estimării rezultatelor studiului, s-a evidenţiat o relaţie cauzală dintre factorii cercetaţi şi starea de sănătate a populaţiei, care consumă apă din apeductul alimentat din r. Prut (tab. 1). În principiu, valorile acestor relaţii nu sunt mari (r=0,2-0,4). Doar indicii mineralizării apei, care se manifestă ca factor de protecţie în etiologia bolilor aparatului circulator, prezintă corelaţii indirecte puternice (r=-0,8), criteriul exactităţii fiind egal cu 3,2. Raportul dintre duritatea apei din apeductul alimentat din r. Prut cu prevalarea bolilor aparatului circulator este mică (r=-0,2, t=0,6).

Tabelul 1. Gradul de corelare dintre unii indici ai calităţii apei din apeduct alimentat din r. Prut şi principalele grupe de nosologii ale morbidităţii prin adresabilitate.

Forme nosologice	Reziduu sec, mg/dm³			Duritate, mmol/dm³			Hidrocarbonaţi, mg/dm³			Mineralizare, g/l		
	r	m	t	r	m	t	r	m	t	r	m	t
a. boli ale sângelui	-	-	-	0,2	0,4	0,5	0,4	0,3	1,3	-	-	-
b. maladii endocrine	-	-	-	-	-	-	0,4	0,37	1,1	-	-	-
c. afecţiuni digestive	-	-	-	0,2	0,3	0,6	0,4	0,37	1,1	-	-	-
d. boli osteoarticulare	0,2	0,3	0,5	-	-	-	0,4	0,37	1,1	-	-	-
e. maladii genito-urinare	0,2	0,3	0,5	-	-	-	0,3	0,37	0,8	-	-	-
f. afecţiuni circulatorii	-	-	-	-0,2	0,4	0,6	0,3	0,4	0,7	-0,8	0,2	3,2

Preponderent, duritatea este determinată de conţinutul sărurilor de Mg, care au manifestat corelaţii directe, mici şi medii, cu bolile sângelui (r=0,42; t=1,1), maladiile sistemului digestiv (r=0,3; t=0,76) şi afecţiunile sistemului genito-urinar (r=0,24; t=0,61). Cu maladiile sistemului circulator prezintă o corelaţie indirectă puternică atât sărurile de Mg, cât şi cele de Ca (respectiv, r=-0,61; t=1,9 şi r=-0,75; t=2,7).

De asemenea, indicele durităţii a manifestat unele corelaţii mici cu bolile sângelui şi cu afecţiunile aparatului digestiv (r=0,2), criteriul exactităţii fiind sub pragul autenticităţii (t=0,5-0,6). Practic aceeaşi legitate se atestă şi la interrelaţiile dintre bolile aparatului osteoarticular şi cele ale sistemului genito-urinar cu concentraţia reziduului fix în apa cercetată (respectiv, r=0,2; t=0,5).

Hidrocarbonaţii au prezentat legături corelative directe medii cu bolile sângelui, maladile endocrine, cele ale aparatului digestiv, aparatului os-

teoarticular (r=0,4; t în limitele 1,1-1,3) şi cu afecţiunile sistemului genito-urinar, bolile aparatului circulator (respectiv, r=0,3, t în limitele 0,7-0,8).

Corelaţii multiple au prezentat indicii calităţii apei din sondele arteziene cu morbiditatea populaţiei. La fel, ca şi în cazul precedent indicele mineralizării sumare dar şi duritatea au manifestat o corelaţie medie indirectă cu bolile aparatului circulator (r=-0,3-0,5 cu t=0,4-0,8), iar concentraţia sărurilor de Mg prezintă o corelaţie indirectă puternică egală cu r=-0,7; t=1,4. O corelaţie directă puternică s-a înregistrat între sărurile de Mg şi Ca cu bolile sângelui (corespunzător, r=0,8-0,9; criteriul exactităţii variind în limitele de 1,9-2,9).

Gradul de mineralizare a apei a manifestat raporturi directe evidente cu bolile sângelui (r=0,9; t=2,9) şi cu afecţiunile endocrine (r=0,8; t=1,9), iar cu maladille sistemului genito-urinar corelaţia este directă mică (r=0,2; t=0,4). O corelaţie directă pregnantă cu bolile sângelui o are şi concentraţia reziduului fix în apa cercetată ((r= 0,9; t=2,9). Un alt indice al mineralizării apei, ∑Na+K, a relevat corelaţii directe puternice, în egală măsură, cu bolile sistemelor digestiv, osteoarticular şi genito-urinar (r=0,7; t=1,4).

Alte grupuri de elemente chimice ale apei, ce s-au manifestat ca factor de risc pentru starea de sănătate a populaţiei din ecosistemul râuliui Prut sunt: hidrocarbonaţii, sulfaţii, clorurile şi azotaţii. În special, hidrocarbonaţii au înregistrat legături directe puternice şi medii cu bolile sistemelor digestiv, osteoarticular, genito-urinar, circulator (r=0,6-0,9; t=1,1-2,9). Aceeaşi legitate, ca şi în cazul hidrocarbonaţilor, cu excepţia bolilor sistemului circulator, dar şi prin valori mai mici, s-a manifestat în ceea ce vizează concentraţia azotaţilor în apă. Conţinutul de cloruri şi de sulfaţi în apă exprimă dependenţe cu bolile sângelui (r=0,5-0,9; t=0,8-2,9). Clorurile au prezentat o relaţie cauzală şi cu afecţiunile endocrine (r=0,7; t=1,4).

Populaţia din localităţile riverane ale Prutului foloseşte pe larg, în scop potabil, apa din fântâni.

În urma analizei indicilor calităţii apei din fântâni, în special, a mineralizării sumare şi a ∑Na+K, remarcăm corelaţii directe puternice şi medii cu bolile sistemului genito-urinar (r=0,8; t=2,5), cu afecţiunilr sângelui (r=0,5-0,6; t=1,04-1,5), cu cele endocrine (r=0,3-0,5; t=0,7-1,2), ale sistemului digestiv (r=0,4-0,5; t=0,8-1,02). Totodată mineralizarea apei a prezentat o corelaţie indirectă puternică cu bolile sistemului circulator (r=-0,7; t=1,9), iar duritatea ei a manifestat corelaţie directă puternică cu bolile sistemului osteoarticular (r=0,7; t=2,1).

Bolile endocrine, bolile sistemului digestiv şi bolile sistemului genito-urinar sunt într-o anumită corelaţie cu concentraţia de hydrocarbonaţi din apa cercetată (respectiv, r=0,3, t=0,5; r=0,5, t=1,2; r=0,6, t=1,5). Bolile sângelui au prezentat o relaţie cauzată de concentraţia reziduului sec din apa din fântâni (r=0,4; t=0,9). În acest sens bolile sistemului osteoarticular (r=0,5) şi bolile aparatului circulator (r=0,3), au manifestat legături directe medii între conţinutul sulfaţilor în apă, criteriul exactităţii fiind sub pragul autenticităţii (t=1,1 şi respectiv, t=0,6). Aceeaşi legitate au prezentat bolile infecţioase şi bolile aparatului osteoarticular cu concentraţia amoniacului dar şi a azotaţilor din apă (r=0,5, t=1,1; r=0,3, t=0,7).

O etapă importantă a cercetărilor, ce are ca scop evidenţierea direcţiilor de activitate şi a măsurilor de prevenţie, constă în determinarea riscului de îmbolnăvire a populaţiei ce utilizează, în scop potabil, apă din surse cu diferit grad de neconformitate cu normele igienice în vigoare.

Iniţial, am analizat riscul de îmbolnăvire a populaţiei după adresabilitate (Forma 12), atât sub aspect teritorial, cât şi conform sursei din care se consumă apă în scop potabil. În acest sens, am analizat riscul de îmbolnăvire a populaţiei din oraşul Lipcani (tab. 2), unde, în scopuri potabile, este consumată apă din sonde şi fântâni, caracterizată prin exces de compuşi ai mineralizării. Lotul de control l-a constituit populaţia ce consumă apă din apeductele alimentate din râul Prut (oraşul Cahul).

Tabelul 2. Riscul de îmbolnăvire a populaţiei din or. Lipcani, care consumă apă neconformă.

Grupe nosologice	RR	Ra	Fae(%)
a. boli osteoarticulare	2,1	0,03	60
b. afeţiuni circulatorii	1,8	0,12	46,1
c. maladii ale sângelui	1,3	0,003	33,3
d. boli digestive	1,3	0,03	33,3
e. afecţiuni genito-urinare	0,8	-0,005	-16,6

Astfel, în funcţie de criteriile nominalizate, pe primul loc putem plasa bolile aparatului osteoarticular, care, la persoanele ce consumă apă neconformă din sondele şi fântânile din or. Lipcani, se atestă mai frecvent de 2,1 ori (RR) decât la populaţia ce foloseşte apă din apeductele alimentate din r. Prut (or. Cahul). De asemenea, riscul atribuibil este Ra=0,03 (Ra<0, factor de protecţie, Ra=0, factor indiferent, Ra>0, factor de risc), ceea ce denotă un factor de risc la grupul de persoane expuse. S-a constatat că utilizarea apei din fântâni şi sonde constituie cauza la 60% (Fae) dintre bolile aparatului osteoarticular, diag-nosticate la populaţia expusă factorului de risc estimat.

Pe locul doi se situează bolile sistemului circulator, care s-au atestat de 1,8 ori mai frecvent (RR) la subiecţii expuşi, comparativ cu grupul de control (Ra=0,12; Fae=46,1%).

Apa din sonde şi din fântâni se caracterizează printr-o duritate mai mare, comparativ cu apa din apeductul alimentat din râul Prut şi ar trebui să se prezinte ca factor de protecţie, însă incertitudinea acestei supoziţii se explică atât prin faptul că oraşul Lipcani se confruntă cu o insuficienţă a personalului medical, cât şi şi prin particularităţile de comportament şi de alimentaţie a populaţiei.

Locul trei, în funcţie de riscul de îmbolnăvire, a revenit bolilor sângelui, care deseori sunt reprezentate de anemii. La persoanele ce consumă, în scop potabil, apă din sonde, dar mai ales din fântâni, unde deseori nivelul de nitraţi depăşeşte nivelul CMA, acestea s-au înregistrat cu o frecvenţă de 1,3 ori (RR) mai mare decât la grupul de control (Ra=0,003; Fae=33,3%). Tot pe locul 3 se plasează şi grupul maladiilor sistemului digestiv, care, la fel, au prezentat o frecvenţă de 1,3 ori (RR) mai mare, respectiv, Ra=0,03; Fae= 33,3%.

Bolile aparatului genito-urinar s-au situat pe locul 4 şi au manifestat o expunere relativ mai mică de 1, deci nu prezintă factor de risc, RR=0,8; Ra= -0,005; Fae=-16,6%. Presupunem că explicaţia ar consta, ca şi în cazul bolilor sistemului circulator, în lipsa unei diagnosticări eficiente.

De asemenea, am analizat riscul de îmbolnăvire a locuitorilor din oraşul Ungheni (tab. 3), unde majoritatea populaţiei foloseşte apa din apeductul alimentat din râul Prut, în scopuri potabile, dar sunt şi dintre cei care consumă apă din fântâni. În lotul de control a fost inclusă şi populaţia din oraşul Cahul, care utilizează apă doar din apeductul alimentat din râul Prut.

În localitatea menţionată, pe primul loc, s-au plasat bolile sângelui, care, la persoanele ce consumă apă neconformă se atestă mai frecvent de 2,9 ori (RR), faţă de populaţia din oraşul Cahul, cu o frecvenţă la cei expuşi de Ra=0,01 şi respectiv, Fae=68,4%. Locul doi le revine maladiilor aparatului digestiv, care se înregistrează cu o frecvenţă mai mare de 1,6 ori la persoanele ce consumă apă din apeduct şi din fântâni decât la cei care beau apă doar din apeduct şi reprezintă un risc de Ra=0,05, la 45,6% de persoane expuse.

Bolile aparatelor circulator, osteoarticular, genito-urinar au prezentat un risc de circa 1,1 ori mai mare (RR), comparativ cu grupul de control şi, respectiv, Ra=0,002-0,005 şi Fae=2,05-12,5.

Tabelul 3. Riscul de îmbolnăvire a populaţiei din oraşul Ungheni, care consumă apă neconformă.

Grupe nosologice	RR	Ra	Fae (%)
a. boli ale sângelui	2,9	0,01	68,4
b. maladii ale aparatului digestiv	1,6	0,05	45,4
c. afecţiuni ale aparatului circulator	1,01	0,003	2,05
d. boli ale sistemului osteoarticular	1,1	0,002	7,6
e. maladii ale aparatului genito-urinar	1,1	0,005	12,5

La următoarea etapă, am determinat riscul de îmbolnăvire a populaţiei după datele din fişele medicale personale, în funcţie de sursa de apă consumată în scopuri potabile. Drept lot de control au servit persoanele ce consumă apă din apeduct alimentat din râul Prut.

În primul caz am studiat posibilitatea îmbolnăvirii populaţiei ce consumă apă din sondele arteziene (tab. 4). În acest sens, cel mai mare risc, de circa 2,1 ori mai mare (RR) faţă de persoanele ce consumă apă din apeduct, îl prezintă bolile aparatului genito-urinar, Ra a acestor maladii la subiecţii expuşi este egală cu 0,17 (Ra), iar 54,8% (Fae) din bolile aparatului genito-urinar diagnosticate la populaţia expusă poate fi explicată prin influenţa factorului de risc estimat.

S-a stabilit că în această grupă de maladii riscul cel mai mare îl prezintă litiaza urinară (RR=1,9; Ra=0,04; Fae=50%) şi pielonefritele (corespunzător, RR=1,7; Ra=0,042; Fae=44,6%).

Tabelul 4. Riscul de îmbolnăvire a populaţiei care consumă apă neconformă din sonde.

Grupe nosologice	RR	Ra	Fae (%)
a. boli ale aparatului genito-urinar	2,1	0,17	54,8
b. maladii ale aparatului osteoarticular	1,7	0,2	41,6
c. afecţiuni ale aparatului digestiv	1,04	0,58	54,2
d. boli ale aparatului circulator	0,89	-0,008	-12,9

Pe locul doi se plasează bolile sistemului osteoarticular, care s-au determinat de 1,7 ori mai frecvent (RR) la subiecţii expuşi, comparativ cu grupul de control (Ra=0,2; Fae=41,6%). Din această grupă de maladii, riscul relativ a fost mai mare pentru artropatii (RR=1,7; Ra=0,09; Fae= 45%) şi pentru osteocondroză (RR=1,3; Ra=0,022; Fae= 25%).

Locul trei, în funcţie de riscul de îmbolnăvire, le-a revenit bolilor aparatului digestiv, care, la persoanele ce consumă apă în scop potabil din sonde, s-a atestat cu o frecvenţă de 1,04 ori mai mare, comparativ cu lotul de control (Ra=0,58; Fae= 54,2%). La acest grup de maladii, cel mai mare risc relativ este specific pentru gastrite şi pentru duodenite (RR=3,7; Ra=0,22; Fae=75,8%), ulcere gastrice (RR=2,6; Ra=0,05; Fae=71,4%), pancreatite (RR=1,7; Ra=0,1; Fae=50%).

La acest compartiment este necesar de menţionat că, în cazul bolilor aparatului circulator, apa din sonde, având un grad de mineralizare mai mare, comparativ cu apa din apeduct, s-a manifestat ca un factor de protecţie. Astfel, am constatat că maladiile aparatului circulator se atestă cu o frecvenţă de 0,8 mai mică la persoanele ce folosesc apa din sonde, comparativ cu cei care consumă apa din apeductele alimentate din râul Prut.

Compoziţia chimică a apei din fântâni se caracterizează printr-un grad de mineralizare mai mare, dar şi prin excesul unor indicatori ai poluării organice.

În continuare s-a analizat riscul de îmbolnăvire a persoanelor ce utilizează în scop potabil apa din fântânile cercetate, în comparaţie cu cei care consumă apa din apeductul alimentat din râul Prut (tab. 5). Cel mai mare risc pentru persoanele ce consumă apa din fântâni îl constituie bolile sângelui, în special anemiile, care se întâlnesc de 2,5 ori mai frecvent (RR), incidenţa fiind cu 0,13 mai mare (Ra). Prin utilizarea apei din fântâni pot fi explicate 65% (Fae) dintre bolile sângelui.

Spre deosebire de persoanele expuse la neconformităţile calităţii apei din sonde, la populaţia ce utilizează apa din fântâni, conform riscului estimat, pe locul doi se plasează bolile aparatului genito-urinar (RR=2,3; Ra=0,19; Fae= 57,5%). Maladiile din grupul menţionat, care cel mai des se atestă la populaţia care consumă apa din fântâni sunt identice ca şi la persoanele ce utilizează apa din sonde.

Pentru persoanele care consumă apa din fântâni, în scopuri potabile, riscul de a contracta o maladie din grupul bolilor aparatului osteoarticular este de 1,6 ori mai mare (RR), comparativ cu persoanele neexpuse la factorul estimat (Ra=0,19; Fae=40,4%). Din acest grup de maladii, riscul cel mai mare persistă pentru osteoporoză (RR=1,9; RA=0,01; Fae=50%), artropatii (RR=1,5; Ra= 0,07; Fae=38,8%) şi osteocondroză (R=1,5; Ra=0,04; Fae=40%).

Tabelul 5. Riscul de îmbolnăvire a populaţiei care consumă apă neconformă din fântâni.

Grupe nosologice	RR	Ra	Fae (%)
a. boli ale sângelui	2,5	0,13	65
b. maladii ale aparatului genito-urinar	2,3	0,19	57,5
c. afecţiuni ale aparatului osteoarticular	1,6	0,19	40,4
d. boli digestive	1,01	0,52	51,4
e. maladii ale aparatului circulator	0,55	-0,32	-84,2

Bolile sistemului digestiv, la persoanele expuse, s-au înregistrat de 1,01 ori mai des (RR) şi de 0,52 mai frecvent (Ra), comparativ cu lotul de control. Factorul estimat constituie cauza a 51,4% dintre maladiile menţionate. Din acest grup de afecţiunii, cel mai frecvent s-au înregistrat ulcere gastrice

(RR=2,6; Ra=0,05; Fae=71,4%), gastrite şi duodenite (RR=2,5; Ra=0,13; Fae=65%), hepatite (RR=2,3; Ra=0,07; Fae=58,3%), pancreatite (RR=2,05; Ra=0,13; Fae=54,1%).

Ca şi în cazul apei din sonde, apa din fântânile investigate se manifestă ca un factor de protecţi pentru bolile aparatului circulator. Astfel, la persoanele care consumă, în scop potabil, apa din fântâni, aceste maladii se atestă de 0,55 ori mai puţin.

DISCUŢII

Conform datelor OMS, în ţările Uniunii Europene, apa potabilă cauzează până la 6% dintre maladii, iar în Republica Moldova, ea reprezintă un factor care determină circa 25-30% dintre bolile somatice. Facilitarea accesului la surse sigure de apă potabilă constituie unul din obiectivele majore ale Protocolului privind Apa şi Sănătatea al Convenţiei din 1992 despre protecţia şi utilizarea cursurilor de apă transfrontalieră şi a lacurilor internaţionale (8).

Rezultatele studiului efectuat corelează cu datele din literatura de specialitate în domeniul dat. Mai multe cercetări au confirmat că aportul scăzut de calciu în organism poate duce la rahitism, coagularea sângelui, fracturi de oase, iar aportul crescut duce la maladii cardiovasculare (9). În acest sens, cercetătorii din Portugalia, efectuând un studiu, au observat o scădere a fracturilor de coapse de 0,30% (95% CI 0,26 la 0,34), 0,25% (95% CI 0,19 – 0,30) şi 0,83% (95% CI 0,81-0,86) odată cu creşterea concentraţiei de 1 mg/l a calciului, magneziului şi de 1 µg/l de fier şi un risc crescut de 26,55% (95% CI 21,17-32,17), 0,17% (95% CI 0,14 la 0,20), 1,53% (95% CI 1,27-1,80) şi 2,64% (95% CI 2,35-2,94) la creşterea cu 1 mg/l a fluorului şi de 1 µg/l a aluminiului, cromului, seleniului; deci calciul, magneziul şi fierul în apa potabilă ajută la prevenirea fracturilor de coapsă, în timp ce fluorul, aluminiul, cromul şi seleniul par a fi un factor de risc [10].

Cercetătorii din Iran, *Abbas B. et al.,* (11), în urma efectuării unui studiu, nu au găsit nicio corelaţie între urolitiază şi concentraţia mare de calciu în apa potabilă, iar magneziul a avut o importanţă semnificativă în incidenţa bolii (p=0,5).

Cercetătorii din regiunea Nantes (Franţa) au studiat dacă există o corelaţie între nivelul turbidităţii apei din apeductul alimentat din râul Loire şi incidenţa gastroenteritelor la copii şi adulţi. Astfel, s-a constatat că la nivelul turbidităţii în apă de (0.042-0.056 NTU) (12) există un risc de 4,2% (CI95=(1,5%; 6,9%)) la copii şi 2,9% (CI95= (0,5%; 5,4%)) la adulţi de a contracta gastroenterite.

CONCLUZII

1. S-a estimat existenţa dependenţelor corelative directe, mici şi medii, dintre indicii calităţii apei din apeductul alimentat din râul Prut şi prevalarea principalelor grupe de nosologii ale morbidităţii. Dependenţe corelative directe mari s-au cuantificat între bolile sângelui şi conţinutul de reziduu sec, duritate, mineralizarea sumară a apei din sondele arteziene (r=0,9), bolile endocrine şi mineralizarea sumară (r=0,8), afecţiunile sistemulor digestiv, osteoarticular, genito-urinar şi ∑Na+K (r=0,7). Concentraţia hidrocarbonaţilor din apa din sonde a corelat direct cu bolile sistemul or digestiv (r=0,6), osteoarticular (r=0,70), genito-urinar (r=0,7), dar şi cele ale sistemului circulator (r=0,9).

2. În cazul populaţiei ce foloseşte apa din sonde, conform valorilor riscului estimat (RR), pe primul loc se plasează bolile aparatului genito-urinar (RR=2,1), locul doi le revine maladiilor sistemului osteoarticular (RR=1,7) şi locul trei – bolilor sistemului digestiv (RR=1,04). Pentru populaţia care consumă apa din fântâni riscul cel mai mare este asociat cu maladiile sângelui/anemiile (RR=2,5), urmate de bolile aparatelor genito-urinar (RR=2,3), osteoarticular (RR=1,6), digestiv (RR=1,01).

CONFLICT DE INTERESE

Autorul n-a declarat conflict de interese.

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Date of receipt of the manuscript: 28/04/2020
Date of acceptance for publication: 15/10/2020

OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

MEDICAL SCIENCES

OPEN ACCESS

ATYPICAL PRESENTATION OF A HIGH-GRADE ASTROCYTOMA IN A PEDIATRIC RHEUMATOLOGY PRACTICE

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DOI: 10.38045/ohrm.2021.1.04 CZU: 616-006.484.03-053.2

Key words: *astrocytoma, brain tumor, musculoskeletal mask, children.*

Introduction. *Hip pain is common in childhood. The main dilemma is to differentiate between benign conditions and those causing significant morbidity and mortality.*

Material and methods. *A clinical case report of a toddler presenting with Musculoskeletal (MSK) mask due to a brain tumor.*

Results. *A 2-year-old boy presented to the ER complaining of hip pain, muscle weakness, and limping. The neurologic exam showed no abnormalities. At the 3rd day of admission, patient condition worsened, and papillary edema was revealed. MRI revealed a tumor in the suprasellar cistern/prepons region.*

Conclusions. *In case of atypical MSK symptoms, a careful evaluation of children should be carried out to prevent any delay in diagnosis.*

Cuvinte cheie: *astrocitom, tumoare cerebrală, mască musculoscheletală, copii.*

PREZENTAREA ATIPICĂ A UNUI CAZ DE ASTROCITOM ÎN PRACTICA CLINICII DE REUMATOLOGIE PEDIATRICĂ

Introducere. *Durerea coxofemurală este frecventă în copilărie, însă esenţial este de a diferenţia afecţiunile benigne de cele care cauzează morbiditate şi mortalitate semnificative.*

Material şi metode. *Vom raporta cazul unui copil de doi ani cu tumoare cerebrală, care a prezentat iniţial diverse acuze din partea sistemului musculoscheletal.*

Rezultate. *Un copil s-a adresat la Departamentul de urgenţă cu dureri în articulaţia coxofemurală, mers şchiopătat, slăbiciuni. Examenul neurologic nu a depistat anumite anomalii. În a 3-a zi starea copilului s-a agravat şi a fost efectuat RMN cerebral, la care s-a identificat o formaţiune a cisternei supraselare/prepontine.*

Concluzii*. În cazul simptomelor musculoscheletice atipice, o evaluare minuţioasă a copiilor este esenţială, pentru a asigura stabilirea cât mai timpurie a diagnosticului.*

INTRODUCTION

Hip pain is quite common in childhood. An important dilemma is to differentiate between benign and self-limiting disorders and those that cause significant morbidity and mortality such as malignant processes (1). This present case report highlights the importance of considering malignancy in the differential diagnosis of childhood hip pain, despite its rare occurrence.

In childhood, brain tumors often involve the musculoskeletal system and mimics and a variety of orthopedic problems at presentation. An accurate anamnesis and physical examination can still be misleading. Moreover, laboratory tests and other investigations are usually inconclusive (2,3).

Thus, additional information is needed to better understand the variety of clinical presentations, inclusively mimickers, diagnostic options, management strategies, postoperative course, and longterm outcomes of patients with malignancies, particularly of those with atypical presentations (4).

MATERIAL AND METHODS

A clinical case of a 2-years-old male patient with musculoskeletal mask due to a brain tumor was reported. The patient was admitted to the Rheumatology clinic from a tertiary level hospital providing pediatric services. We will highlight its peculiarities in correlation with available literature data from PubMed/NCBI, Medline, Hinari with an emphasis on clinical and imaging features that may aid in diagnosis. The literature review was based on the analysis of the published case presentations, synthesis and reviews on the following key words: astrocytoma, brain tumor, musculoskeletal mask, children.

RESULTS

A 2-year-old boy presented to the emergency department by self-addressing. He felt sick for the last 2 days, presenting an acute upper respiratory infection with catarrhal signs at the onset of clinical symptoms and no fever episodes. At the 3rd day of the disease, he started to complain of painful left hip and difficulties on walking. Parents were worried and referred to the hospital. There was no previous relevant medical history, any chronic disease or similar past episodes. The child's physical development corresponded to his age: weight at the 71 percentiles (+0.56 SDS according to WHO2000 standards), height at the 86 percentiles (+1.09 SDS), head circumference at the 80 percentiles (+0.84 SDS). Moreover, none neurological milestones abnormalities were revealed from his history taking, neither in the medical outpatient documentation. On physical examination, he walked with a limp, but due to pain exacerbation he refused to walk by himself. Movements of the left hip were painful (mainly external rotation), but not restricted. He complained of muscle weakness and, consequently, difficulty to sit up alone, as well as to maintain after getting help. Also, he complaint of mild dysuric symptoms, mainly during micturition. During the exam he was very moody, irritable and excessively cried.

The emergency department organized a multidisciplinary approach, involving a large team from different units like pediatric neurology, orthopedics, pediatric surgeon, pediatric urologist and pediatric rheumatologist. The neurology exam didn`t show any abnormalities at that time – no data for neuropathy or focal signs.

Further laboratory tests did not reveal any abnormalities: no anemic syndrome, all acute phase reactants (ESR, CRP, fibrinogen) were within normal range. Biochemical serum tests also didn`t show any possible underlying causes – normal values for muscle enzymes, liver and renal tests. An initial X-ray to the pelvis revealed no changes. An ultrasound of the left hip was performed, revealing small infusion.

During the follow-up, at the 3rd day of admission, our patient presented repeated matinal vomiting and worsening of previous signs – muscle weakness, missing gross motor milestones, which were previously present, ataxia, an excessive crying when being in orthostatic position, superficial sleeping time. On repeated neurological and ophthalmological examination, an abnormal optic disc appearance with papillary edema and a marked venous congestion was reported. Thus, the following step of the diagnostic approach was the brain MRI 1.5 T with contrast. MRI revealed a volume mass tumor of 58*47*47mm within the suprasellar cistern/prepons region, with a solid non-homogenous component and cystic appearance, compressing the 3rd ventricle and, respectively, with acute obstructive biventricular hydrocephaly (fig.1).

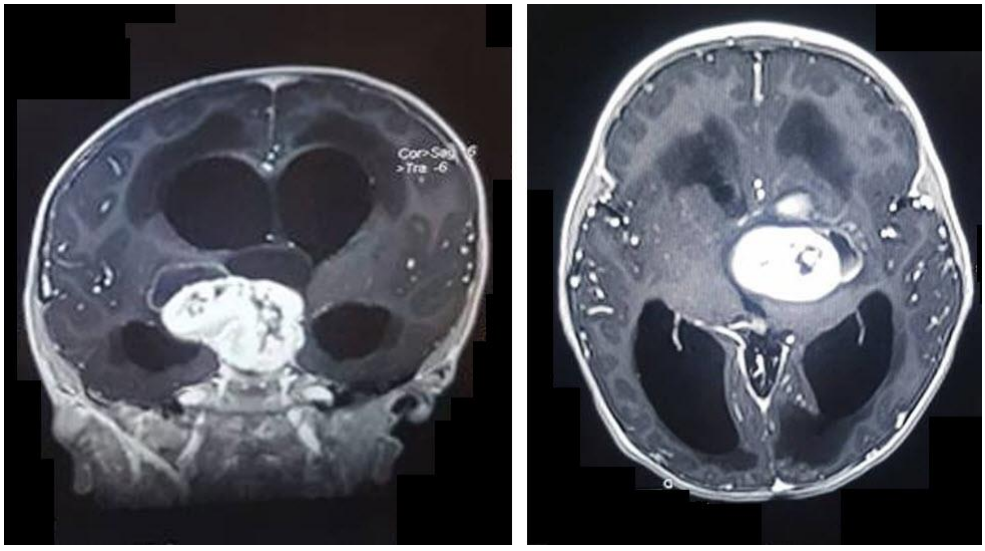

Figure 1. Preoperative coronal and axial postcontrast T1- Weighted MRI images.

The patient was transferred to pediatric neurosurgery department where he underwent several surgical interventions. Figure 2 shows the 2nd MRI image following the first intervention, which carried out decompression via a shunt through the right ventricle. However, the tumor increased in volume at a 7-day interval. It increased to 64*98*55 mm with invasion of the 3rd ventricle and left lateral ventricle.

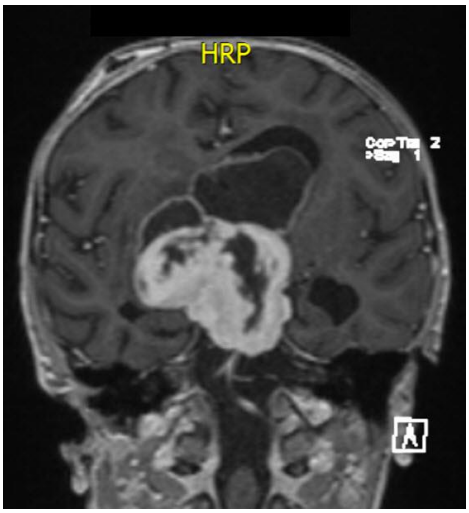

Figure 2. Postoperative (1st step), coronal postcontrast MRI.

Another surgical intervention was performed in order to get the tumor biopsy. The histological exam established a high-grade astrocytoma, IV WHO degree of malignancy.

At 6 months after the onset of the disease, our patient continued chemotherapy and radiotherapy in the oncology pediatric department. Additionally, the patient's follow-up was carried out within the neurosurgery clinic.

DISCUSSIONS

Nowadays, primary brain tumors are the leading cause of cancer-related mortality in children and adolescents aged below 20 years, now surpassing leukemia (5). Figure 3 shows the structure and incidence of CNS tumors among children (3).

Astrocytoma is the foremost neuroglial tumor occurring throughout infancy and childhood and is derived from and composed of astrocytes showing varying degrees of differentiation (6). These tumors usually present within the first 2 years of life, most often by 4-6 months of age. Males are more commonly affected than females (1.5-1.7:1), and patients commonly present with rapidly enlarging head circumference and, some-times, vomiting and seizures (4).

Brain tumors in children can present with many different signs and symptoms, largely dependent on the location of the tumor (7). Headache, nausea, vomiting, and vision loss can be caused by increased intracranial pressure due to obstruction of cerebrospinal fluid flow by tumor growth. Visual field deficits and hormone deficiencies can be presenting signs of suprasellar masses (3). Ataxia and clumsiness can be caused by posterior fossa tumors, and seizures or personality changes can result from tumors involving cortical areas, particularly in frontal brain regions (3).

According to current classification, there are low- and high-grade astrocytoma. Those ones differ significantly in terms of their histological grading, site of origin, treatment, and prognosis (6). Figure 4 represent a brief presentation of astrocytomas distribution among pediatric population (5).

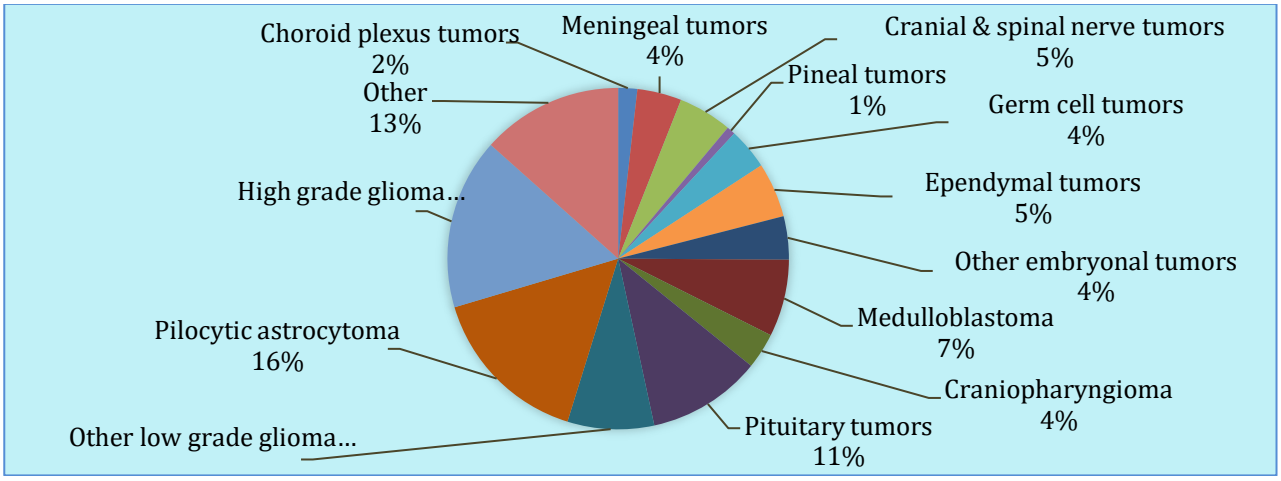

Figure 3. Primary CNS tumors in children, 0-18 y.o. (Segal D., 2016) (3).

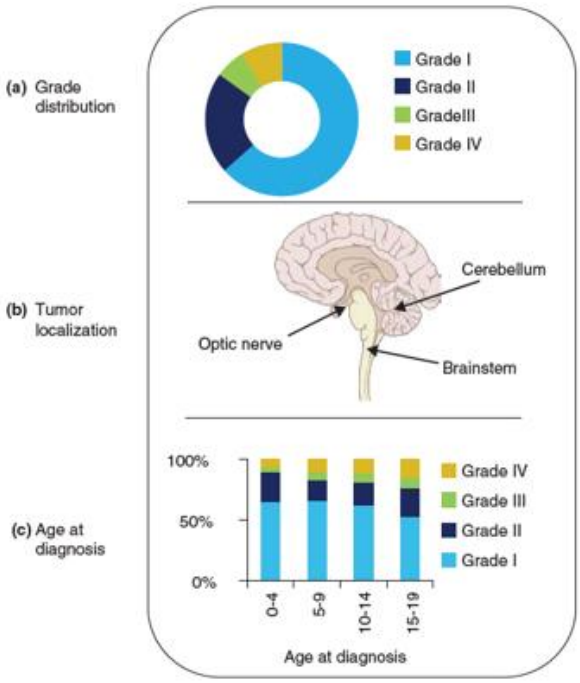

Figure 4. Overview of the pediatric astrocytomas: grade distribution, tumor localization, age at diagnosis (Gerges N., 2013) (5).

The WHO's grading system is based on histologic criteria and is used to determine prognosis, select treatment, and stratify patients for research studies.

The present case study represent a 4th degree of astrocytoma, which highlights the specific features as (5):

- *Histology* – Infiltrative, nuclear atypia, high mitotic activity, pseudo-palissading necroses, florid microvascular proliferation.
- *Location* – Cerebrum, cerebellum and spine, but brainstem glioblastoma multiforme (DIPGs) are more frequent in children.
- *Prevalent age group* – Adults, except a *diffuse intrinsic pontine glioma*, which is prevalent in children below 10 years of age.
- *Therapy* – Surgical resection when possible, fol-lowed by adjuvant radiation and chemotherapy.
- *Prognosis* – Very poor (1.2% 5-year survival).

In general, for CNS tumors, the 5-year survival rates vary widely with tumor type and have improved greatly with improved surgical and oncologic care. At present, the overall 5-year survival rate for children aged 0–19 years after diagnosis with a CNS tumor is estimated to be73.6% (3). Many studies have indicated that, as a group, malignant gliomas in children and young adults carry a more favorable prognosis than do comparable lesions in older patients (8).

Due to the infiltrative nature of these tumors, however, even extensive surgical resection will leave malignant cells in the region. Many high-grade gliomas occur in highly sensitive brain areas, such as the thalamus or pons, where even a limited resection may be impossible (3).

Ongoing studies aim to illuminate new avenues to address drug resistance and to identify novel molecularly targeted agents that may work synergistically, finally providing improved therapeutic options in *diffuse intrinsic pontine glioma* and other diffuse midline gliomas with genetic mutations (9). Researchers are discovering that the genetic and epigenetic characteristics of a tumor are far more instructive in helping to predict its behavior and choose optimal therapies (3). The molecular revolution for pediatric brain tumors has begun, and one can envision an era of highly specialized therapeutics will follow (10).

CONCLUSION

1. Our findings highlighted the therapeutic dilemmas that arise with musculoskeletal mimics, as well as the importance of thorough investigation to distinguish mimickers from true neoplasms. This case emphasizes not only the need for a careful assessment of children with persistent or atypical hip pain but also encourages the enriched knowledge and practical experience sharing.

2. Any medical diagnosis should take a multimodal approach, as entities that mimic neoplasms have overlapping features and may present detrimental outcomes if they are underdiagnosed.

3. Despite being a rare presentation of brain tumor, the delay in the diagnosis may have devastating consequences.

CONFLICT OF INTERESTS

All authors declare no competing interests.

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Date of receipt of the manuscript: 26/06/2020
Date of acceptance for publication: 30/11/2020

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OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

BIOLOGICAL SCIENCES

OPEN ACCESS

ANTI-ADHESION PROPERTIES OF AMINOPROPANOL DERIVATIVE WITH N-ALKYLARYL RADICAL KVM-194 AGAINST *PSEUDOMONAS AERUGINOSA*

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DOI: 10.38045/ohrm.2021.1.05 CZU: 579.841.11

Key words: *Pseudomonas aeruginosa, hydrophobicity, motility, adhesion, biofilms.*

Introduction. *The present study assessed Pseudomonas aeruginosa surface characteristics, motility and adhesion properties under the influence of 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride (KVM-194).*

Material and methods. *The clinical strain P. aeruginosa 449 was used in the study. The cell surface hydrophobicity (CSH) was evaluated by adhesion to solvent (MATS test). Swimming, swarming and twitching motility of P. aeruginosa were studied by standard methods in media with different agar contents. Cells ability to adhere to polystyrene was assessed by the Christensen method. The effect of KVM-194, meropenem and ciprofloxacin on hydrophobicity and motility was evaluated both at 0.5 or 2.0 minimal inhibitory concentrations (MIC), while on adhesion abilities – only 0.5×MIC.*

Results. *It was shown that 0.5× MIC KVM-194 reduced CSH of P. aeruginosa (by 16%, $p<0.05$), affected swimming motility, and decreased its adhesion to polystyrene. The most pronounced changes in adhesion properties were recorded after 3-5 hours of pre-treatment with this compound. Moreover, it was proven that sub-MICs of meropenem and ciprofloxacin did not alter bacterial cells hydrophobicity and had no significant influence on P. aeruginosa motility and adhesion properties.*

Conclusions. *The present study suggested that KVM-194 affected the initial steps of P. aeruginosa biofilm formation and thus had tremendous potential for new antibiofilm agents' development.*

Cuvinte cheie: *Pseudomonas aeruginosa, hidrofobicitate, mobilitate, aderență, biofilme.*

PROPRIETĂȚI ANTI-ADERENTE A DERIVATULUI AMINOPROPANOL CU RADICAL N-ALCHILARIL KVM-194 ÎMPOTRIVA *PSEUDOMONAS AERUGINOSA*

Introducere. *Prezentul studiu a evaluat caracteristicile suprafeței, mobilitatea și proprietățile de aderență a Pseudomonas aeruginosa, sub influența clorurii de 1-[4-(1,1,3,3-tetrametil-butil) fenoxi]-3-(N-benzil-hexametilenimin)-propan-2-ol (KVM-194).*

Material si metode. *În studiu au fost utilizate 449 de tulpini clinice de P. aeruginosa. Hidrofobicitatea suprafeței celulare (CSH) a fost evaluată prin aderență la solvent (testul MATS). Mobilitatea, roirea și mișcarea bacteriilor P. aeruginosa au fost studiate prin metode standard în medii cu conținut diferit de agar. Capacitatea celulelor de a adera la polistiren a fost evaluată prin metoda Christensen. Efectul KVM-194, meropenemului și ciprofloxacinei asupra hidrofobicității și mobilității a fost evaluat atât la concentrații inhibitorii minime de 0,5 sau 2,0 (CMI), cât și asupra abilităților de aderență – doar 0,5×CMI.*

Rezultate. *S-a demonstrat că 0,5×CMI KVM-194 a redus CSH-ul P. aeruginosa (cu 16%, %, $p<0,05$), a afectat mobilitatea și a redus aderența la polistiren. Cele mai pronunțate modificări ale proprietăților de aderență au fost înregistrate după 3-5 ore de pre-tratament cu acest compus. De asemenea, s-a arătat că sub-CMI-urile meropenemului și ciprofloxacinei nu au modificat hidrofobicitatea celulelor bacteriene, nu au avut nicio influență semnificativă asupra mobilității și proprietăților de aderență ale P. aeruginosa.*

Concluzii. *Prezentul studiu sugerează că KVM-194 acționează la etapele inițiale de formare a biofilmului de către P. aeruginosa și, prin urmare, are un potențial enorm pentru dezvoltarea de noi preparate antibiofilm.*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen, characterized by resistance to a wide range of antibacterial drugs and the ability to form biofilms. Microbial community is irreversibly associated with biotic (tissue, wounds) and abiotic surfaces (intravascular, urethral catheters, various metal grafts, contact lenses) (1). Biofilm formation leads to an increase both in the duration of hospitalisation and in the persistence of infectious disease. It could also cause fatal complications of catheter-associated infections. Adhesions, proliferation, formation of microcolonies are conditions required for effective colonization of favourable surface. Adhesion is provided by various motility modes (2). The bacterial cell surface hydrophobicity (CSH) is one of the factors that determines the strength of adhesion (3, 4).

The migration and dispersion provide *P. aeruginosa* with potential advantages while searching for sources of nutrients, avoiding negative environmental factors, access to optimal colonization and distribution sites in the environment, etc. (5). Bacteria of the genus *Pseudomonas* can move via several motility modes on environments with various viscosities. Swimming motility is derived by flagella that allows bacteria to disperse in liquid environments (6, 7). Type IV pili are involves twitching motility over the top of solid surfaces (2). Flagella and pili, as well as production of rhamnolipids, provide swarming motility in viscous environment of the lungs with cystic fibrosis, which is as closely as possible mimicked *in vitro* by semi-solid agar (5, 8, 9). It is remarkable that, in the same conditions, cells can also exhibit a sliding movement that does not require pili or flagella (8).

Some antibiotics at subinhibitory concentrations (sub-MICs) can affect the colonization of surfaces by microorganisms, via disturbing the biofilm formation processes. Thus, β-lactam antibiotics such as penicillins and cephalosporins reduce the cell hydrophobicity of gram-positive (*S. aureus*) and gram-negative (*P. aeruginosa*) bacteria (3, 10). At the same time carbapenems, other β-lactam antibiotics show no effect on CSH, motility and adhesion properties (10). Sub-MICs of tobramycin cause no changes in swarming motility of *P. aeruginosa* (11) but reduce this parameter in *E. coli* (7). The macrolide antibiotic azithromycin also inhibits this mode of motility due to impaired synthesis of rhamnolipids, which are surfactants (12).

Investigation of the mechanisms of various surfaces colonized by bacteria, as well as evaluation of antimicrobials effect on the initial stages of biofilm formation, are essential for further deve-lopment of preventive strategies against biofilm formation, as well as for solving the problems of ineffective antimicrobial therapy for infections caused by microbial communities.

Previously, we had found that 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride (KVM-194) showed a broad spectrum of antibacterial activity (13), among which – *in vitro* activity against biofilms of gram-negative and gram-positive bacteria (14, 15, 16). As KVM-194 possessed membranotropic effects and affected the composition of cell walls of gram-negative bacteria (17), we hypothesized that this compound could affect bacterial cell surface properties and adhesion to polystyrene.

The purpose of this present paper was to study the influence of 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride on adhesion properties of *Pseudomonas aeruginosa*.

MATERIAL AND METHODS

Bacterial strains and subculture conditions

The bacterial strain used in the present study was *Pseudomonas aeruginosa* 449 isolated from pus. The test strain showed resistance to cefepime and tetracycline, intermediate susceptibility to ceftriaxone, cefotaxime and meropenem, susceptibility to aztreonam, cefoperazone, ciprofloxacin, amikacin, and gentamicin. The strain was subcultured at 37°C on Tryptone Soya Agar plates.

Antimicrobials, chemicals, and media

The 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride (KVM-194) used in the present study was synthesised within the Institute of Organic Chemistry of NAS of Ukraine. KVM-194 was dissolved in 10% dimethyl sulfoxide; the stock solution concentration was 1 mg/mL. All other chemicals were obtained from commercial sources.

Ciprofloxacin and meropenem were purchased in the pharmacy under the trade name Ciprinol (CIP, solution for infusion, manufactured by KRKA, Slovenia) and Meronem (MER, powder for solution for injection, manufactured by AstraZeneca UK Limited, United Kingdom) were used as comparator agents. The following media were used in the

present study: Luria-Bertani broth (Conda, Spain), Luria-Bertani agar and Tryptone Soya Broth (TSB, HiMedia, India).

Bacterial surface hydrophobicity assay

P. aeruginosa surface hydrophobicity was measured using previously described microbial adhesion to solvents (MATS) method with modifications (18). The affinity to ethyl acetate that is a monopolar and basic solvent was studied. An overnight culture in TSB medium was 10-fold diluted with fresh TSB medium. The hydrophobicity properties were estimated by growing strain in TSB (optical density OD_{600} 0.3) with or without KVM-194 (25 μg mL^{-1}), MER (1.0 μg mL^{-1} or 4.0 μg mL^{-1}) or CIP (0.125 μg mL^{-1} or 0.5 μg mL^{-1}) at 37°C for 90 min. After incubation, bacteria were washed twice in 0.9% NaCl solution by centrifugation for 15 min at 3000 rpm and were resuspended in same solution to OD_{600}0.18-0.22 (A_0). Afterwards, ethyl acetate (0.5 mL) was added to the bacterial suspensions (3.0 mL), which were then kept at room temperature (RT) for 10 min to saturate. Each sample was then mixed by vortexing (model V-3, ELMI, Latvia) for 2 min and then allowing the mixture to stand for 15 min at room temperature for phase separation. The aqueous phase was collected and the OD_{600} was measured (A). The results were expressed as the percentage decreased in the OD of the aqueous phase (A) compared with the OD of the initial cell suspension (A_0): $100\times[1-(A/A_0)]$. Each assay was repeated three times in duplicate.

Motility assay

The swarming, swimming, and twitching motilities of *P. aeruginosa* were investigated using the following media: (I) swim plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.3% agar], (II) swarm plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% agar, 1M $MgSO_4$, 0.5% glucose], and (III) twitch plates [1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.0% agar]. An overnight cell culture in TSB medium was incubated 30-45 min with 0.5 or 2.0×MIC KVM-194 (25.0 μg mL^{-1} and 100 μg mL^{-1} respectively), meropenem (1.0 μg mL^{-1} and 4.0 μg mL^{-1} respectively), ciprofloxacin (0.125 μg mL^{-1} and 0.5 μg mL^{-1} respectively). Control cultures contained no antimicrobials. Each assay was repeated three times in duplicate.

For the *swimming motility assay*, the plates were inoculated in the centre with a sterile toothpick and incubated for 16-20 h at RT (10). Motility was assessed by observation of the circular turbid zone formed by bacteria migrating away from the inoculation point.

For the *swarming motility assay*, the bacterial cells were gently inoculated by micropipette (2 μL) into the top of semisolid agar, and the plates were incubated at 37°C for 16-24 h (19).

For the *twitching motility assay*, the cells were stabinoculated with a sterile toothpick through an agar layer to the bottom of the Petri dish. After incubation for 24-48 h at 37°C, a hazy zone of growth at the interface between the agar medium and the glass surface was observed. The ability of bacteria to twitch strongly on the glass surface was examined by removing the agar, washing out the untouched cells and staining the attached cells by a crystal violet solution (10).

Adhesion assay

The adhesion of *P. aeruginosa* was estimated by the method by Christensen (20). An overnight culture in TSB medium was grown at 37°C in the presence or absence of 0.5× MIC KVM-194 (25 μg mL^{-1}), meropenem (1.0 μg mL^{-1}) or ciprofloxacin (0.125 μg mL^{-1}). After 1, 3, 5 or 7 h, the bacterial cells were diluted 100-fold with fresh TSB medi-um; cell suspension (100 μL) was transferred into individual wells of sterile, polystyrene, 96-well plate and incubated at 37°C. After a 24-hour incu-bation, the TSB medium was discarded, and the wells were washed thrice with distillate water to remove nonadherent bacteria. Adherent cells were fixed in place for 15 min with 96% ethanol, dried and then stained for 5 min with 0.1% crystal violet. Excess stain was rinsed off. After drying, the optical densities (OD) of stained adherent bacterial films were measured using Absorbance Microplate Reader (model ELx800, BioTek, USA) at 630 nm. Adherence measurements were re-peated at least three times in quadruplicate; the values were then averaged.

The adherence capability of the test strain was classified into four categories: non-adherent, slightly adherent, moderately adherent, or strongly adherent, based upon the OD of bacterial films. The cut-off optical density (ODc) was defined as three standard deviations above the mean OD of the negative control. The strength of adhesion was calculated by the following formula: $OD \leq ODc$ – non-adherent; $ODc < OD \leq 2\times ODc$ – slightly adherent; $2\times ODc < OD \leq 4\times ODc$ – moderately adherent; $4\times ODc < OD$ – strongly adherent.

Statistical Analysis

The obtained data were expressed as means±standard deviation (SD). The nonparametric Kruskal-Wallis H-test was used to compare the continuous variables. A p-value of <0.05 was considered as significant. STATISTICA, version 10 (StatSoft, USA) was used for the data analysis.

RESULTS

Effect of KVM-194 on Pseudomonas aeruginosa surface hydrophobicity

The cell surface hydrophobicity is an important physical factor at the stage of surface attachment, which determined the strength of adhesion. Particularly, bacteria with a hydrophobic surface adhere better than with a hydrophilic one (3, 4), which allow them to colonize the tissues and surfaces of medical devices, form biofilms and exacerbate the course of the infection process.

The present study investigated the influence of KVM-194, meropenem and ciprofloxacin on the hydrophobic properties of *P. aeruginosa*. The data reported in Table 1 showed that KVM-194 at 0.5× minimal inhibitory concentration (MIC) reduced bacterial hydrophobic properties by 16% compared to the intact control ($p < 0.05$). High turbidity of medium was observed in the presence of KVM-194 at 2.0×MIC, which caused erroneous results (data not shown).

Table 1. The effect of KVM-194, meropenem or ciprofloxacin on MATS of *P. aeruginosa* depending on concentration.

Antimicrobials	Solvent affinity (%, mean±SD) to ethyl acetate	
	0.5×MIC	2.0×MIC
KVM-194	52.77±2.492*	N/A
Meropenem	60.23±3.499	60.86±1.196
Ciprofloxacin	61.05±0.316	58.55±0.971
Control (without antimicrobials)	62.64±2.905	

** in comparison with control (bacterial growth without antimicrobials) p<0.05.*

Both Meropenem concentrations did not alter *P. aeruginosa* 449 cells hydrophobic properties. The same results were obtained for ciprofloxacin.

Effect of KVM-194 on Pseudomonas aeruginosa motility

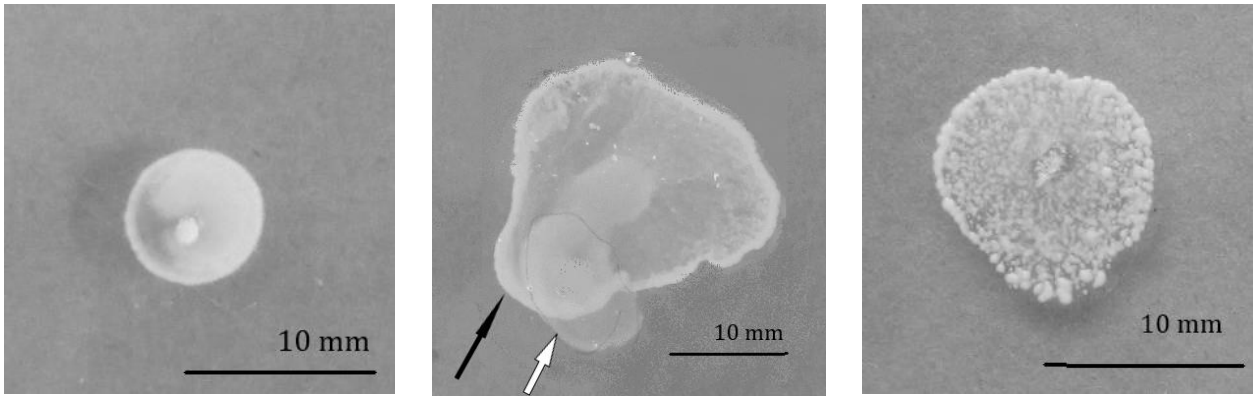
Single gram-negative bacteria cells swim in a liquid environment using flagella, which allow *P. aeruginosa* to respond to attractants and repellents. For solid surfaces or human body tissues, colonization pili are required (2), which are also responsible for intercellular aggregation (21). Type IV pili allow cells to move on the top of solid surface by extension and retraction of filaments (2).

Flagella and type IV pili are essential for swarming; these structures are involved in colonization of the mucus. Swarming is initiated when cell density reached a certain threshold (8, 22). Strains of *P. aeruginosa* with altered swarming motility are defective in biofilm formation that indicates its important role during early stages of biofilm development. Conversely, strains with a swarming phenotype were more resistant to antibiotics (ciprofloxacin, gentamicin, polymyxin) (11). In general, changes in *P. aeruginosa* motility properties are correlated with impairment in biofilm formation (23).

The present study assessed the effect of KVM-194, meropenem, and ciprofloxacin on the movement of *P. aeruginosa* within or on the top of media surface. It was found that cell movement was dependent on the presence of flagella (swimming), type IV pili (twitching), as well as their combination (swarming): the diameters of corresponding motility zones were 7.0±0.05 mm, 9.0± 2.28 mm and 10.7±1.03 mm (fig. 1).

In subsequent experiments, the effect of different concentrations (0.5×and 2.0×MIC) of KVM-194, meropenem, and ciprofloxacin on different modes of *P. aeruginosa* motility was studied (tab. 2).

KVM-194 sub-MIC affected swimming. The diameter of motility zone was reduced by 46% compared to the control ($p<0.05$). The higher concentration of KVM-194 (2.0×MIC) completely inhibited the motility. Meropenem reduced swimming zone of the culture by 14% compared to the control ($p<0.05$), the effect was not dose-dependent. Ciprofloxacin did not affect swimming of *P. aeruginosa*.



A B C

Figure 1. Swimming (A), twitching (B) and swarming (C) motility in *P. aeruginosa*.
Colonies of strain P. aeruginosa 449 from a TSB were inoculated, as described in Material and Methods. The black arrow shows the edge of colony on the agar surface; the white arrow shows the edge of colony between the agar and glass surface.

Table 2. The effect of KVM-194, meropenem or ciprofloxacin on motility of *P. aeruginosa* depending on concentration.

Antimicrobials	Motility (mm, mean±SD)					
	Swimming		Twitching		Swarming	
	0.5×MIC	2.0×MIC	0.5×MIC	2.0×MIC	0.5×MIC	2.0×MIC
KVM-194	3.8±0.98 #, **	ND	12.5±9.09	ND	7.0±1.79 **	ND
Meropenem	6.0±0.05 #	6.0±0.05 #	12.2±7.22	ND	11.5±4.14	ND
Ciprofloxacin	6.7±0.52	6.7±0.52	14.2±13.12	ND	14.7±3.88	ND
Control (without antimicrobials)	7.0±0.05		9.0±2.28		10.7±1.03	

ND – non-detected, complete inhibition of growth
 # – in comparison with control (bacterial growth without antimicrobials) p<0.05.
*** – in comparison with the same concentration of ciprofloxacin p<0.05.*

Complete inhibition of cell growth and twitching motility was observed under the influence of 2.0×MIC of KVM-194 or antibiotics. With sub-MICs, the twitching zones of the culture acquired a more elongated shape compared to the control samples; however, no significant differences were found (p>0.05).

KVM-194 2.0×MIC completely inhibited the swarming motility. The motility zones diameter reduction of *P. aeruginosa* under the influence of KVM-194 sub-MIC was also registered, however, the changes were not significant compared to control samples (p>0.05). Meropenem and ciprofloxacin also inhibited the motility and growth of *P. aeruginosa* at a concentration of 2.0×MIC. Pre-treatment with sub-MIC of antibiotics led to swarming zones induction, however, there was no statistically significant difference compared to control samples (p>0.05).

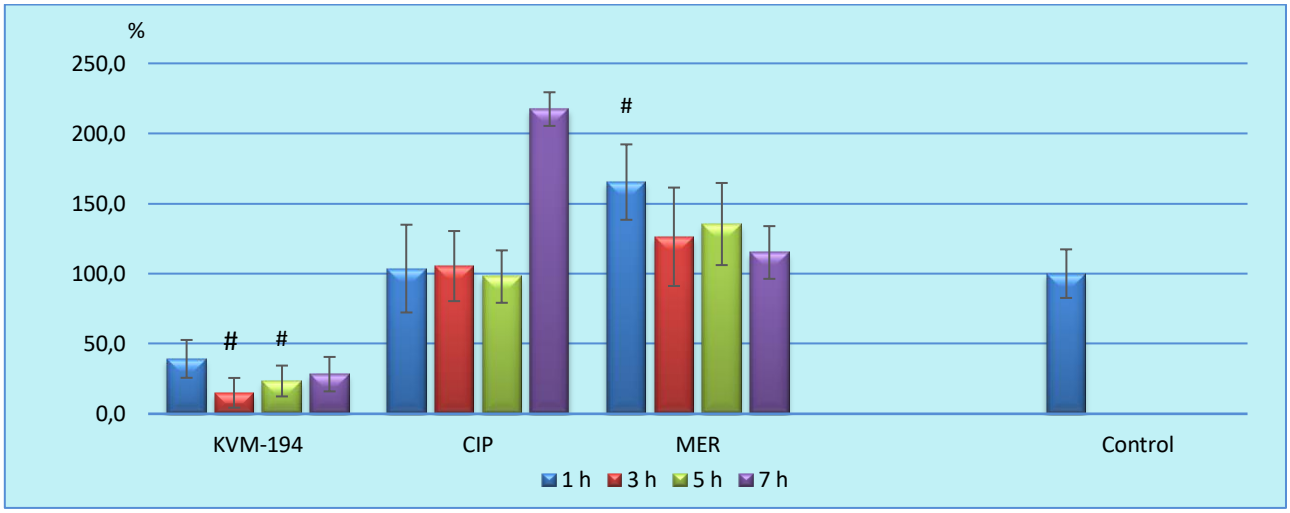
Thus, KVM-194 at subinhibitory concentration reduced the swimming motility of *P. aeruginosa*; at higher concentration (2.0×MIC); inhibition of all motility patterns was observed. It could be assumed that KVM-194 largely affected the motility of *P. aeruginosa* due to flagella rather than to type IV pili. Meropenem and ciprofloxacin at concentration of 2.0×MIC inhibited the growth, twitching and swarming motility of *P. aeruginosa*, however, both antibiotics showed no effects on motility under the influence of sub-MIC. The reduction in the swimming zone was noted under the influence of meropenem. Ciprofloxacin at a subinhibitory concentration did not affect this bacterial motility mechanism.

Effect of KVM-194 on adhesion of Pseudomonas aeruginosa

P. aeruginosa 449 was a slightly adherent strain. The influence of time of pre-treatment with KVM-194, meropenem and ciprofloxacin on adhesion ability of *P. aeruginosa* was compared with the intact control sample (100%) (fig. 2). The experiments showed that KVM-194 decreased adhesion of *P. aeruginosa* cells by 60.8–85.0%, the most pronounced changes occurring after 3 and 5 h of incubation.

Meropenem and ciprofloxacin did not decrease

the adhesion of *P. aeruginosa* cells; moreover, there was a significant increase in adhesion of bacteria after 1 hour of incubation in the presence meropenem compared to the control sample (p<0.05).



 # - in comparison with control (bacterial growth without antimicrobials) p<0.05.

Figure 2. Influence of 0.5×MIC of KVM-194, meropenem (MER) or ciprofloxacin (CIP) on adhesion (%) of *P. aeruginosa* grown until exponential phase.

DISCUSSION

Short-term contacts of *P. aeruginosa* with the surfaces of tissue cells or medical devices lead to the bacterial attachment and biofilm production. An important physical factor at this stage is the hydrophobicity of the surface of bacterial cells, which determines the strength of adhesion, especially when the surface of the substrate shows a marked hydrophobic property (3, 4).

The present study showed that pre-treatment of *P. aeruginosa* cells with KVM-194 (0.5×MIC) decreased CSH that might cause reduction of cell adhesion to the surface. According to other authors' data, penicillins and cephalosporins at subinhibitory concentrations also decreased CSH of *P. aeruginosa* (3, 10).

Ciprofloxacin and meropenem showed no influence on CSH. These results were confirmed by another study, which described no *P. aeruginosa* hydrophobicity changes induced by sub-MIC imipenem (10).

The ability to colonize surfaces could lead to biofilm formation (23). *P. aeruginosa* cell generates a single polar flagellum, which helps in moving through liquid environments (8). Flagellum and chemoreceptors respond to attractants and repellents, which allows bacteria to detect the appropriate substrate for colonization. On the top of solid surfaces, microorganisms can move by twitching, powered by the extension and retraction of type IV pili (2, 6, 7). Pili are also important for attachment to epithelial cells and abiotic surfaces (2).

When cells transited from swimming to swarming due to environmental viscosity increase, *P. aeruginosa* may produce two polar flagella. Swarming is initiated when cell density reaches a certain threshold (8, 22). This motility pattern is regulated by Las and Rhl-systems of intercellular communication (*Quorum sensing*) (5). This movement pattern is also associated with the regulation of virulence factor genes, particularly, *lasB* (elastase activity) and *pvdQ* (pyoverdine biosynthesis) (11). Additionally, swarming plays an important role during early stages of biofilm formation, because the non-motile strains are defective in biofilm formation (11).

The obtained data suggested that KVM-194 largely affected the motility of *P. aeruginosa* due to flagella (than type IV pili). Both KVM-194 concentrations caused considerable changes in swimming motility. According to other researchers, inhibition of swimming and twitching motility was also common to piperacillin/tazobactam (10). Another motility pattern was affected by azithromycin, which inhibited the swarming motility of *P. aeruginosa* via impairment of rhamnolipids synthesis (12).

The obtained data suggested that KVM-194 largely affected the motility of *P. aeruginosa* due to flagella (than type IV pili). Both KVM-194 concentrations caused considerable changes in swimming motility. According to other researchers, inhibition of swimming and twitching motility was also common to piperacillin/tazobactam (10). Another motility pattern was affected by azithromycin, which inhibited the swarming motility of *P. aeruginosa* via impairment of rhamnolipids synthesis (12).

It is known that aminoglycoside antibiotics at subinhibitory concentrations increase the expression of regulators for various motility genes (24), and strains with a swarming phenotype are characterized by a decrease in sensitivity to ciprofloxacin, gentamicin, and polymyxin. However, the effect of subinhibitory concentrations of gentamicin and tobramycin on swarming motility of *P. aeruginosa* has not been established (11). Carbapenems (imipenem) do not affect swimming and twitching motility (10). According to our results, meropenem and ciprofloxacin at subinhibitory concentration had practically no effect on the examined motility patterns of *P. aeruginosa*.

A decrease in the hydrophobic properties of the cell surface and inhibition of motility in the presence of piperacillin/tazobactam led to a disruption of surface attachment (10). Similar results were obtained when studying the KVM-194. It was also found that carbapenems (meropenem) and fluoroquinolones (ciprofloxacin) in sub-MICs caused no changes in the cell surface hydrophobicity ans had no significant impact on the motility and adhesion capacity of *P. aeruginosa.*

CONCLUSIONS

1. The present study showed that 1-[4-(1,1,3,3-tetra methyl butyl) phenoxy]-3-(N-benzyl hexa methylene iminium)-2-propanol chloride is a promising candidate for further development of new agents for preventing biofilm formation.

2. The KVM-194 at subinhibitory concentration (0.5×MIC) decreased the cell surface hydrophobicity of *P. aeruginosa* (by 16%, $p<0.05$) and its motility via flagellum (swimming), which led to reduction of *P. aeruginosa* attachment to polystyrene. The most remarkable changes in adhesion properties were recorded after 3 to 5 hours of pre-treatment with this compound.

CONFLICT OF INTERESTS

Authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by Ukrainian National Academy of Medical Sciences (№ 0118U001559).

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Date of receipt of the manuscript: 17/07/2020
Date of acceptance for publication: 12/12/2020

OH.RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

BIOLOGICAL SCIENCES

THE LICHEN GENUS *LEPRARIA* ACH. (STEREOCAULACEAE, LICHENIZED ASCOMYCOTA) IN ROMANIA

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DOI: 10.38045/ohrm.2021.1.07 CZU: 582.29(498)

Key words: *lichenized fungi, Lepraria, key, biodiversity.*

Introduction*. The genus Lepraria includes diffuse or indefinitely delimited species, always sterile, Lendemer (1) naming it „the most unusual member of the sterile lichen crusts". According to Tonsberg (2), the leprarioid state appeared as an adaptation to a substrate characterized by dry surfaces, in sites with high humidity and low illumination. The thallus is leprose with an entirely sorediate surface. The lack of fruiting bodies has made the lichens from this group among the least known and studied for a long time. The increase in the number of studies on sterile crustose lichens in recent period has led to an increase in the number of accepted species of the genus Lepraria. The information on the genus Lepraria is not uniformly distributed, the current checklist describes only two species, L. caesioalba and L. finkii in Romania. The recent appearance of several studies that indicate the presence in Romania of other species of the genus mentioned, created the motivation of this study.*
Material and methods. *This study is based on material from the Babes-Bolyai University (CL) herbaria in Cluj-Napoca, Romania and literature data. For 13 species, specimens have been examined.*
Results. *The key for the species of genus Lepraria is proposed, adapted from Wirth (3). The species distribution maps in Romania are given.*
Conclusions. *Fifteen Romanian species are revised, Lepraria eburnea, L. ecorticata, L. umbricola were reported from two locations, L. neglecta was found only in one location. Most widely distributed species in Romania seems to be L. finkii and L. membranacea.*

Cuvinte cheie: *ciuperci lichenizate, Lepraria, cheie de determinare, biodiversitate.*

GENUL DE LICHENI *LEPRARIA ACH.* (STEREOCAULACEAE, ASCOMICETE LICHENIZATE) ÎN ROMÂNIA

Introducere. *Genul Lepraria include specii cu tal difuz sau delimitat nedefinit, întotdeauna sterile, Lendemer (1) numindu-l „cel mai neobişnuit membru al lichenilor crustoşi sterili". După Tonsberg (2), starea leprarioidă a apărut ca o adaptare la un substrat caracterizat prin suprafeţe uscate, în staţiuni cu umiditate ridicată şi iluminare redusă. Talul este lepros cu o suprafaţă în întregime sorediată. Lipsa corpurilor de fructificaţie a făcut ca, pentru multă vreme, lichenii din acest grup să fie printre cei mai puţin cunoscuţi şi studiaţi. Creşterea numărului de studii asupra lichenilor crustoşi sterili în ultima perioadă, a dus la creşterea numărului de specii din genul Lepraria acceptate. Informaţiile despre genul Lepraria nu sunt distribuite uniform, lista actuală descrie doar două specii, L. caesioalba şi L. finkii în România. Apariţia recentă a mai multor studii care menţionează prezenţa în România a altor specii din genul menţionat, a creat motivaţia acestui studiu.*
Material si metode*. Acest studiu se bazează pe materialul lichenic din Herbarul Universităţii Babeş-Bolyai (CL Herbarium) din Cluj-Napoca, România şi datele din literatura de specialitate. Pentru 13 specii, au fost examinate eşantioane.*
Rezultate*. Cheia pe care o propunem este adaptată după Wirth (3). Deasemenea, sunt date hărţile de distribuţie a speciilor în România.*
Concluzii. *Cincisprezece specii din România sunt revizuite, Lepraria eburnea, L. ecorticata, L. umbricola au fost raportate din două locaţii, L. neglecta a fost găsită doar într-o singură locaţie. Cele mai răspândite specii din România se consideră a fi L. finkii şi L. membranacea.*

INTRODUCTION

The genus *Lepraria* includes diffuse or indefinitely delimited species, always sterile, Lendemer (1) naming it "the most unusual member of the sterile lichen crusts". The thallus is leprose with an entirely sorediate surface (4). Molecular studies indicates gender affiliation to Stereocaulaceae familly (4). The lack of fruiting bodies makes lichens in this group among the least known and studied (1). The leprarioid state appeared as an adaptation to a substrate characterrized by dry surfaces, in sites with high humidity and low illumination (2). However, since 2000 there was an increase in the number of studies on sterile crustose lichens (5), which also increased the number of taxa of the genus *Lepraria* accepted. Thus, Lendemer (1) documents the existence of 57 species and 2 varieties worldwide. The information on the genus *Lepraria* is not uniformly distributed, Ciurchea's checklist (6) describes only two species of the genus *Lepraria* in Romania, respectively *L. caesioalba* and *L. finkii*. The recent appearance in Romania of several doctoral thesis (7, 8, 9) and scientific articles that mention the presence of other species of the genus mentioned above, create the motivation of this study. We established a list of 15 species, for which we made a key attempt.

In Romania, *Lepraria* taxa stands in beech forests, coniferous and mixed forest, dwarf mountain pine shrubs and alpine meadows. Species of *Lepraria* can inhabit bark, wood, soil or rock, and frequently in sheltered sites where they are protected from direct sunlight.

MATERIAL AND METHODS

This study is based on material from the Babes-Bolyai University (CL) herbaria in Cluj-Napoca, Romania and literature data. For 13 species, specimens have been examined by specialists from: *Lepraria caesioalba* – Department of Botany, Institute of Botany, Academy of Sciences Pruhonice (10), *Lepraria lobificans* – Herbarium Université de Liège (LGHF) and Swiss Federal Research Institute WSL (11); *L. nivalis* – Herbarium Hungarian Natural History Museum (BP) and Swiss Federal Research Institute WSL (11); *L. jackii, L. rigidula, L. vouauxii* – Department of Botany, Institute of Botany, Academy of Sciences Pruhonice (10), Swiss Federal Research Institute WSL (11); *Lepraria eburnea, L. ecorticata, L. elobata, L. membranacea, L. negelecta, L. toensbergiana, L. umbricola* – Swiss Federal Research Institute WSL (11). The key we propose is adapted from Wirth (3). The species distribution maps in Romania are given.

RESULTS

Lepraria species distribution in Romania

1. *Lepraria caesioalba* (de Lesd.) J.R. Laundon var. ***caesioalba*** (fig. 1)
Lichenologist 24: 324 (1992); *Crocynia caesioalba* de Lesd., Bull. Soc. Bot. France 61: 84 (1914); *Leproloma caesioalba* (de Lesd.) M. Choisy, Bull. mens. Soc. linn. Lyon II 19: 12 (1950) *Crocynia henrici* de Lesd., Bull. Soc. Bot. France 61: 84 (1914).
Alba County: Detunata Mt. (12).
Arges County: Iezer-Papusa Mts., Riusorul to Zanoaga Mt. (9).
Brasov County: Tampa Mt. (12).
Hunedoara County: Retezat Mts., alpine zone around the lake Bucura (10).

2. *L. eburnea* J.R. Laundon (fig. 2)
Lichenologist 24: 331 (1992); *Lepraria frigida* J.R. Laundon, The Lichenologist 24: 332 (1992).
Maramures County: Rodnei Mts., Borsa (CL) 664515; Rodnei Mts., Repedea Valley (CL) 664494, 664518.

3. *L. ecorticata* (J.R. Laundon) Kukwa (fig. 3)
Mycotaxon 97: 64 (2006); type: United Kingdom, England, Devon, Ilfracombe, Torrs Walks, 1. Sept. 1971, J. R. Laundon 2851 (BM—holotypus). *Lecanora ecorticata* J.R. Laundon, Nova Hedwigia 76: 100 (2003).
Maramures County: Rodnei Mts., Pietrosul Mare Nature Reserve (CL) 664498; Rodnei Mts., Repedea Valley (11).

4. *L. elobata* Tonsberg (fig. 4)
Sommerfeltia 14: 197 (1992)
Maramures County: Rodnei Mts., Cascada Cailor (CL) 664508; Rodnei Mts., Izvorul lui Dragos Valley, Pietrosul Mare Nature Reserve, Repedea Valley (11).

Figure 1. Distribution of *Lepraria caesioalba* var. *caesioalba* in Romania.

Figure 2. Distribution of *Lepraria eburnea* in Romania.

Figure 3. Distribution of *Lepraria ecorticata* in Romania.

Figure 4. Distribution of *Lepraria elobata* in Romania.

5. *L. finkii* (Hue) R.C. Harris (1985) (fig. 5)
Crocynia finkii B. de Lesd., in Hue, *Bull. Soc. bot. Fr.* 71: 334 (1924); *Crocynia aliciae* Hue, *Bull. Soc. bot. Fr.* 71: 333 (1924); *Crocynia americana* de Lesd., *Bull. Soc. bot. Fr.* 71: 333 (1924).
Arges County: Iezer-Papusa Mts., Riusorul to Zanoaga Mt. (9).
Bacau County: Casin River Basin, Casin Monastery, Halos Ciubotaru Forest (13).
Bihor County: Bihorului Mts., Rachita Peak (14).
Bistrita-Nasaud County: Rodnei Mts., the upper basin of the Rebra Valley (15); forests near Arcalia Scientific Resort (16); Nasaudului Hills, Satului Valley (17).
Botosani County: Dorohoi surroundings (18).
Cluj County: Gilau-Muntele Mare Mts., Huza Valley (17); Iara Valley (15); Vladeasa Mts., Racad Valley (15); Nonei Valley (19).
Harghita County: Bicazului Gorge (20).
Hunedoara County: Retezat National Park (22), Zlatuia Valley (21).
Ilfov County: Bucharest, Mogosoaia Forest (12).
Maramures County: Barjaba (19).
Prahova County: Ciucas Mts. (23).
Suceava County: Calimani Mts., Draglele Valley (12); Rarau Mts., Pietrele Doamnei (12); Codrul Secular Slatioara (12).

6. *L. incana* (L.) Ach. (fig. 6)
Meth. Lich.: 4 (1803); type: United Kingdom, drawing in Dillenius, Hist. Musc.: tab. I fig. 3 (1742) (holotypus); typotypus: herb Hist. Musc.: tab. I no 3 (OXF). *Byssus incana* L., Sp. Pl. 2: 1169 (1753).
Alba County: Trascau Mt., Detunata (24); Aiud Bichis, Raristi, Lopadea, Valisoara Forest (25).
Arad County: Zarandului Mts., Slatina de Mures, Barzava, Milova (26).

Cluj County: Vladeasa Mts., Poienita, Capra, (24); Gilau Mt., Baisoara (24); Marisel, Feleac (27).
Maramures County: Barjaba (24).
Suceava County: Rotunda Pass (11).

Figure 5. Distribution of *Lepraria finkii* in Romania.

Figure 6. Distribution of *Lepraria incana* in Romania.

7. *L. jackii* Tonsberg (fig. 7)
Sommerfeltia 14: 200 (1992); type: Norway (BG – holotype; BM – isotype); *Lepraria toensbergiana* Slav.-Bay & Kukwa, *Bryologist* 108: 132 (2005).
Hunedoara County: Retezat Mts., camping site Pietrele (10).
Maramures County: Rodnei Mts., Cascada Cailor (CL) 664492, 664501; Rodnei Mts., Repedea Valley, Pietrosul Mare Nature Reserve, Borsa (11).

8. *L. lobificans* Nyl. (fig. 8)
Flora 56: 196 (1873); type: France (H – lectotype; BM – topotype).
Bihor County: Padurea Craiului Mts., Sighiles Valley (1996), between Calatele and Varciorog, (CL) 655943.
Maramures County: Rodnei Mts., Repedea Valley (CL) 664511; Rodnei Mts., Pietrosul Mare Nature Reserve, Borsa (11).
Suceava County: Calimani Mts., Driglele (28); Calimani Mts, Calimani National Park, Haitii Peak, valley of the stream Tarnita (BP) 93460 (29).

9. *L. membranacea* (Dicks.) Vain. (fig. 9)
Acta Soc. Fauna Flora Fennica 49(2): 265 (1921); type: United Kingdom, Scotland, J. Dickson (BM ex K ex D Turner – holotypus). *Lichen membranaceus* Dicks., Fasc. Pl. Crypt. Brit. 2: 21 (1790). *Leproloma membranaceum* (Dicks.) Vain., Term. Füz. 22: 293 (1899).
Alba County: near Campeni, Detunata Mt. (12).
Brasov County: Racatau Valley, Racota Valley, Persani Mts., Dopcei Cave, Bogatii Cave (12).
Cluj County: near Turda (12), Fantanele Dam, Somesului Cald Valley, between Fantanele Dam and Tarnita (30), Vladeasa Mts., Nonei valley, Preluca Rabului (19).
Dambovita County: Bucegi Mts., Zanoagei Gorge (12), Leaota Mts., Cheii Valley (31).
Harghita County: Harghita Mts., Sfanta Ana Lake (12).
Hunedoara County: Retezat Mts., Raul Mare Valley (12), Rausor Valley, Stanisoara Valley, Valeriasca Valley, (12), Retezat National Park, Gemenea Lake, Taul Negru, Zlatuia Valley (21); Cetatea de Boli, near Petrosani, Sureanului Mts., Auselul Mt. (12).
Maramures County: Rodnei Mts., Repedea Valley (11).
Prahova County: Busteni, Urlatoarea Mare, Ialomitei Valley, Sinaia (12).
Sibiu County: Fagaras Mts., Arpas Mt., Cartisoara Mt., (12).
Suceava County: Codrul Secular Slatioara (12).
Valcea County: Lotrului Mts., Calinesti Valley (32), Defileul Cozia (Oltului Valley between Proeni and Calinesti) (33).

Figure 7. Distribution of *Lepraria jackii* in Romania.

Figure 8. Distribution of *Lepraria lobificans* in Romania.

10. *L. neglecta* (Nyl.) Erichsen (fig. 10)
Erichsen, in Lettau, Feddes Repert. 61: 127 (1958); *Lecidea neglecta* Nyl., Not. Skällsk. Fauna Fl. Fenn. Förh. 4: 233 (1859).
Maramures County: Rodnei Mts., Pietrosul Mare Nature Reserve (CL) 664498.

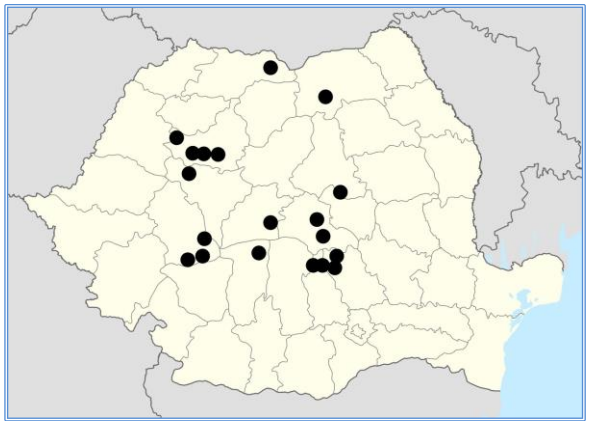

Figure 9. Distribution of *Lepraria membranacea* in Romania.

Figure 10. Distribution of *Lepraria neglecta* in Romania.

11. *L. nivalis* J.R. Laundon (fig. 11)
Lichenologist 24: 327 (1992); *Crocynia murorum* de Lesd., Bull. Soc. Bot. France 95:199 (1948).
Maramures County: Rodnei Mts., Pietrosul Mare Nature Reserve (CL) 664482; Rodnei Mts., Borsa, Repedea Valley, Batrana (11).
Bihor County: Bihor Mts., Zgurasti Cave System (Avenul Zgurasti), 1,5 km NE of Garda de Sus village, between V. Garda Saca and V. Ordancusa (CL) 658547, 658546.

12. *L. rigidula* (de Lesd.) Tonsberg (fig. 12)
Sommerfeltia 14: 205 (1992); type: United Kingdom, Scotland, Perth, Pitlachry, byside of R. Tummel, Jun. 1914, J. McAndrew (E – holotypus). *Crocynia rigidula* de Lesd., in Hue, Bull. Soc. Bot. France 71: 331 (1924).
Hunedoara County: Retezat Mts., alpine zone around the lake Bucura (10).
Maramures County: Rodnei Mts., Cascada Cailor (CL) 664502; Rodnei Mts., Repedea Valley, Borsa (11).

13. *L. toensbergiana* Bayerova & Kukwa (fig. 13)
Bryologist 108(1): 132 (2005); type: Poland, Western Carpathians, Eastern Tatra Mts, Dolina Roztoki Valley, near Nowa Roztoka, 21. Aug. 1999, W. Fałtynowicz, s.n. (UGDA – holotypus; herb. S. Bayerová – isotypus).
Maramures County: Rodnei Mts., Repedea Valley (CL) 664517; Rodnei Mts., Pietrosul Mare Nature Reserve, Izvorul lui Dragos Valley (11).

Figure 11. Distribution of *Lepraria nivalis* in Romania.

Figure 12. Distribution of *Lepraria rigidula* in Romania.

14. *L. umbricola* Tonsberg (fig. 14)
Sommerfeltia 14: 206 (1992); type: Norway, Hordaland, Bergen, Milde, Brandaneset, 25. Sept. 1990, T. Tonsberg 13635 (BG – holotypus).
Maramures County: Rodnei Mts., Izvorul lui Dragos Valley (CL) 664496; Rodnei Mts., Repedea Valley (11).

15. *L. vouauxii* (Hue) R.C. Harris (fig. 15)
Harris, in Egan, The Bryologist 90: 163 (1987); *Crocynia vouauxii* Hue, Bull. Soc. Bot. France 71: 392 (1924); *Leproloma vouauxii* (Hue) J.R. Laundon, The Lichenologist 21: 13 (1989) *Crocynia arctica* Lynge, Skr. om Svalbard og Ishavet 81: 19 (1940); *Lepraria arctica* (Lynge) Wetmore, Publs Mich. St. Univ. Mus., biol. ser., 3: 440 (1968).
Hunedoara County: Campu lui Neag, Cheile Butii, in the gorge Cheile Scorota (10).
Maramures County: Rodnei Mts., Borsa (CL) 664495; Repedea Valley (11).

Figure 13. Distribution of *Lepraria toensbergiana* in Romania.

Figure 14. Distribution of *Lepraria umbricola* in Romania.

Figure. 15. Distribution of *Lepraria vouauxii* in Romania.

Key to the species of *Lepraria* in Romania

1 Thallus sharply bordered at the margin, coherent, in the case of well-developed rounded rosettes, appearing lobed or with radial folds 2
1* Thallus not rounded rosetted and lobed or with radial folds 9
2 On limestone, soil, mosses..... 3
2* On silicate rock, acidophytic mosses, soil, very rarely on bark..... 4
3 Thallus whitish, cream colored, gray-white, pale greenish, folded skin-like, often regularly rounded, often with radially aligned cracks, almost somewhat lobed, in the center often breaking up..... ***L. nivalis***
3* Thallus greenish white, blue-greenish white, thick, coherent, sometimes producing a lobed thallus, at times loosening itself and easily breaking away..... ***L. lobificans***
4 Thallus sharply delimited, at least at the margin pale yellowish, spongy-pliant, of thick coalescing soredia-like, cottony to rather compact granules, with age often undifferentiated leprose. Pannaric acid, roccellic acid ..... ***L. membranacea***
4* Thallus relatively unclearly margined, not yellowish at the margin, not spongy-pliant, on rain exposed habitats on mosses, rarely directly on silicate rock, of relatively compact granules, without pannaric acid 5
5 Atranorin and fumarprotocetraric acid or stictic acid complex. ..... ***L. caesioalba*** var. **caesioalba**
5* Atranorin, alectorialic or angardianic acid..... 6
6 Thallus of coarsely granular, non-powdery soredia containing alectorialic acid ..... ***L. neglecta***
6* Thallus of fine, powdery soredia or with long projecting hyphae, not coarsely granular, containing atranorin acid, rangiformic and angardianic acids always absent, other fatty acids present 7
7 Thallus of soredia with long projecting hyphae, containing atranorin, nephrosteranic acid ..... ***L.rigidula***
7* Thallus of finer soredia without any or only with short projecting hyphae, containing atranorin and fatty acids other than nephrosteranic acid 8
8 Thallus containing atranorin, jackinic acid, and roccellic acid; from lowlands to montane belt ..... ***L. jackii***
8* Thallus containing atranorin and toensbergianic acid; montane, rare at low elevations ..... ***L. toensbergiana***
9 Thallus white..... 10
9* Thallus greenish..... 11
10 Thallus white to light grey, often creamy or slightly greenish, with alectorialic acid, KC+ fleeting! Reddish (observe under the binocular, „scrape" the thallus with a preparation needle)..... ***L. eburnea***
10* Thallus whitish-cream colored, often slightly yellowish, without Alectorialic acid, not KC+ reddish. With Pannaric acid-6-methylester, ... ***L. vouauxii***
11 Thallus leprose..... ***L. elobata***
11* Thallus crustose..... 12
12 With divaricatic acid..... ***L. incana***
12* Without divaricatic acid..... 13
13 Soredia well separated from each-other..... ***L. ecorticata***
13* Soredia often aggregating 14
14 Soredia aggregating in large clusters (up to 200 µm), usually with long projecting hyphae, up to 0,1 mm long..... ***L.finkii***
14* Soredia up to 60 µm, relatively densely packed, projecting hyphae rarely present..... ***L. umbricola***

DISCUSSION

Lepraria caesioalba var. *caesioalba* was found in Romania on mosses growing on smooth bark (*Betula, Alnus*) and on rocks. It belongs to the *L. neglecta* group, being differentiated by the fact that it rarely develops directly on rocks (2). Distribution: Europe, North and South America, Asia, Australasia, Antarctica, Greenland (5).

Lepraria eburnea was found in Romania on dead wood and spruce bark, usually it grows on stone, rarelly on bark (34). Specimens of *L. eburnea* may

be morphologically similar to *L. finkii*, but differ chemically (30). Distribution: in Europe, North America, Asia and Australia (34).

Lepraria ecorticata was found in Romania in *Pinus mugo* shrubs, on bark of *P. mugo*; Kukwa (34) indicates that it prefers rocks. Rare species, reported from Europe, Asia, South America (34).

Lepraria elobata in Romania was identified on dead wood, generally it is found on soil, siliceous rocks and mosses (5). Chemical similarity with *L. lobificans*, which differs morphologically by thallus lobate (2) and cottony appearance (5). Similar chemistry with *L. finkii* which has a wooly, thick thallus (34). Distribution: Europe, North America, Greenland (5).

Lepraria finkii is in Romania a corticolous species, found in coniferous and broadleaf or mixed forests. Similar chemistry with *L. elobata,* species from which it differs morphologically by its wooly, thick thallus (34). Distribution: in all continents, except Antarctica (34).

Lepraria incana is also corticolous in Romania, found in orchards and mixed forests. A species variable morphologically, sometimes similar to *L. elobata,* which differs chemically, lacking divaricatic acid (5). Distribution: all continents, except Antarctica, Arctic regions and North America (34).

Lepraria jackii was found in Romania on spruce bark; it shows in our country similar ecology with specimens growing in Belarus and Poland (34). Distribution: Europe, North America, Asia, Australia (5).

Lepraria lobificans grows in Romania on beech and spruce bark, also on acid soil. For chemically similar species see the discussion under *L. elobata*. Distributed worldwide (5).

Lepraria membranacea was found in Romania on beech and spruce bark. Might be confused with *L. vouauxii* which has pannaric acid 6-methylester (4). Distributed worlwide.

Lepraria neglecta was found in Romania on the soil of alpine pastures. *L. eburnea* has similar chemistry with *L. neglecta*, but has different morphology – thallus granular and coarse – and ecology, living in exposed places to sun and rain (34). Distribution: Europe, North and South America, Australasia, Antarctica, Greenland (5).

Lepraria nivalis was identified on soil and on limestone; in Europe is common in Mediterranean areas and south of the continent (5). It was found also in North America, Australasia, Antarctica, Greenland (5).

Lepraria rigidula in Romania was identified on beech bark. Some specimens could have a greenish colour resembling *L. jackii* (34), which has a different chemistry – atranorin, zeorin and fatty acids (5). Distribution: Europe, North America, Asia, North of Africa (35).

Lepraria toensbergiana was found in Romania on deadwood. Close in morphology to *L. jackii* (which contains jackinic acid), chemically similar to *L. rigidula,* whose soredia have long hyphal projections. Also, distribution differs from *L. jackii* and *L. rigidula, L. toensbergiana* being common in mountain areas (36).

Lepraria umbricola was found in Romania on deadwood. May be found on bark, rock, mosses and soil, especially on acidic substrate. Similar to green coloured *L. ecorticata*, differs by producing thamnolic acid (5). Distribution: Europe, Macaronesia (35).

Lepraria vouauxii in Romania was discovered on deadwood. Occurring worldwide in natural and urban habitats (34).

CONCLUSIONS

1. Fifteen species are included in this study, *Lepraria eburnea, L. ecorticata, L. umbricola* were reported from two locations, *L. neglecta* was found only in one location. Although they do not appear on the Red Lists in Romania or surrounding countries, these species may be endangered in Romania.

2. Most widely distributed species in Romania seems to be *L. finkii* and *L. membranacea*.

3. We subscribe to the statement of Bungartz (37), that identification keys for these lichens typically rely more on chemical characters, morphological characters being considered somewhat problematic.

CONFLICT OF INTERESTS

There are no conflicts of interest to be mentioned.

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Date of receipt of the manuscript: 18/07/2020
Date of acceptance for publication: 19/12/2020

Florin CRISAN, Web of Science ID researche V-1240-2017

OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

BIOLOGICAL SCIENCES

OPEN ACCESS

PECULIARITIES OF EXTRACTION OF β-LACTOGLOBULINE IN PROTEIN MINERAL CONCENTRATES AT ELECTROACTIVATION OF WHEY

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DOI: 10.38045/ohrm.2021.1.06 UDC: 637.344.8

Key words: *whey, protein-mineral concentrates, β-lactoglobulin, electroactivation.*

Introduction. *Whey is a by-product and an excellent source of proteins that is rather aggressive due to a large amount of organic substances it contains. The electro-activation of whey applied in the experiments is a wasteless method that allows the valorification of all whey components. β-lactoglobulin (β-Lg) makes up 50% of the whey proteins and 12% of the total protein content in milk.*

Material and methods. *The recovery of β-Lg in protein-mineral concentrates (PMC) by electro-activation processing of different types of whey with different initial protein content was investigated in seven configurations. The recovery of protein fractions in the PMCs were analyzed via electrophoresis with sodium dodecyl sulfate (SDS-PAGE) and 15% non-denaturing polyacrylamide gel (PAAG).*

Results. *Whey electro-fractionation and the obtaining of PMCs with predetermined protein content, namely of β-Lg, were studied on three whey types, processed at different treatment regimens and in seven configurations. The proper management of electroactivation by varying the treatment regimens will allow the electro-fractionation of different types of dairy by-products.*

Conclusions. *The maximum amount of β-Lg recovered in PMCs on electroactivation is 66-71% depending on the processed whey and on the treatment regimens. Obviously, the extraction of β-Lg from initially lower protein content shows a higher recovery degree of β-Lg. The registered temperatures allows formation of PMCs without thermal denaturation.*

Cuvinte cheie: *zer, concentrate proteice, minerale, β-lactoglobulină, electroactivare.*

PARTICULARITĂȚILE EXTRAGERII β-LACTOGLOBULINEI ÎN CONCENTRATE PROTEICE MINERALE LA ELECTROACTIVAREA ZERULUI

Introducere. *Zerul, ca unul dintre subprodusele lactate, fiind o sursă excelentă de proteine, se prezintă, de asemenea, ca un produs agresiv, din cauza substanțelor organice deținute în cantități mari. Electroactivarea zerului este o metodă non-reziduală care permite valorificarea tuturor componentelor din zer. β-lactoglobulina (β-Lg) reprezintă 50% din proteinele din zer și 12% din conținutul total de proteine ale laptelui.*

Material și metode. *Extragerea β-Lg în concentrate proteice minerale (CPM), la electroactivarea diverselor tipuri de zer, cu un conținut proteic initial diferit, a fost cercetată în 7 configurații. Fracțiile proteice extrase din zer în CPM au fost analizate prin electroforeză cu dodecil sulfat de sodiu (SDS-PAGE) și concentrația gelului de poliacrilamida ne denaturant (PAAG) de 15%.*

Rezultate. *Electrofracționarea zerului și obținerea CPM cu un conținut proteic predeterminat, și anume cu β-Lg, a fost demonstrată la prelucrarea a trei tiputi de zer, în regimuri de tratament diferite, cercetate în 7 configurații. Gestionarea corectă a electroactivării, cu variația regimurilor de tratament, permite electrofracționarea diferitelor subproduse lactate.*

Concluzii. *Cantitatea maximă de β-Lg, extrasă în CPM la electroactivare, constituie 66-71%, în funcție de zerul procesat și de regimurile de tratare. Este evident că volumul de extragere a β-Lg din zerul cu un conținut proteic inițial mai mic, în CPM este mai mare. Temperaturile înregistrate permit formarea CPM fără denaturare termică.*

INTRODUCTION

Primary milk processing allows the production of different types of cheese, of large quantities of various secondary dairy products: whey, buttermilk, skim milk, caseinate, etc., with various solid contents. Global whey production is estimated at about 180-190·10^6 tons/year, of this quantity only 50% is processed. Development of wasteless technologies and processing of whey in a closed cycle is one of the major global challenges (1).

Whey is a by-product and an excellent source of proteins that is also aggressive due to a large amount of organic substances it contains. The manufacture of healthy and environmentally safe whey products requires an upgrade of methods and techniques for whey processing. The electroactivation of whey is a wasteless method that allows the valorification of all whey components. Besides, this type of processing allows controlling the whey protein content in the obtained concentrates, depending on the processing regime (2).

It is known that whey proteins make up about 20% of milk proteins. The four major whey protein components, namely β-lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and immunoglobulin (Ig) make up 90% of whey proteins. The remaining 10% are proteins such as lactoperoxidase, serum transferrin, lactoferrin, lactolin, and proteo-peptone fraction (fig. 1).

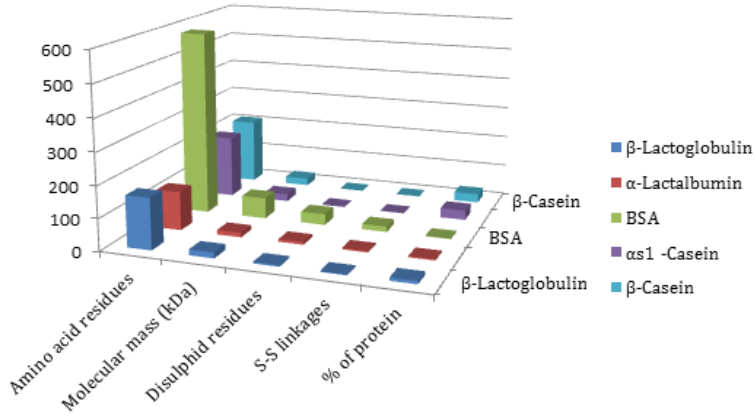

Figure 1. Composition and features of some major milk protein fractions (3).

Whey proteins are well structured proteins with secondary and tertiary stable structures. The main forces involved in assembling and maintaining the globular tertiary structure are disulfide bonds, hydrophobic interactions, hydrogen bonds, and the Van-der-Waals interactions (4).

Whey proteins are highly soluble in milk at a wide range of pH values due to the amino acid composition and the free radicals arrangement during formation of tertiary structures.

This is because of the arrangement of hydrophilic free radicals and a large number of disulphide bonds on the surface of whey proteins with globular structures (5). However, the globular structure induces resistance to proteolysis of those proteins. The four main whey protein fractions are presented below.

β-lactoglobulin. β-lactoglobulin (β-Lg) makes up 50% of the whey proteins and 12% of the total protein content of milk. Native β-Lg is a small globular protein with a molecular mass of 36.6 kDa with defined secondary and tertiary structures. The β-Lg molecule consists of an α-helix, β-sheet and random coil structures represented in a ratio of 10 to 15%, 43% and 47%, respectively (6-8). In aqueous solutions at pH 5-7 and room temperature, this protein is a dimmer of two identical subunits. Each subunit has a molecular mass of 18.3 kDa and is formed of 162 amino acids, of which 84 are essential amino acids and four cysteine residues. Cysteine (a semi-essential proteinogenic amino acid, a component of β-Lg) forms the disulfide bonds at Cys66-Cys160 and Cys106-Cys119 levels (9, 10).

In addition, β-Lg contains a free sulfhydryl SH group (Cys 121) within the native protein, which becomes active after the protein denaturation by various agents (including heating) and can interact with other proteins through disulfide bonds (6, 10-13). These interactions occur at pH 7 and at temperature range of 60-65°C. The free SH group of Cys121 is the most reactive, thus initiating the interchange of intermolecular reaction

with Cys66-Cys160 forming a new S-S bond between Cys121-Cys160, Cys121-Cys66, and Cys160-Cys160, resulting with a new dimmer that contains a new reactive SH group. The new reactive SH group initiates the formation of polymers through S-S bridges interacting with other monomers and oligomers. Cys160 is also active and initiates the interchange reactions SH/S-S, probably, due to its location near C terminus, which makes it more accessible for intermolecular reactions once the S-S bond is broken (fig. 2) (9, 14, 15).

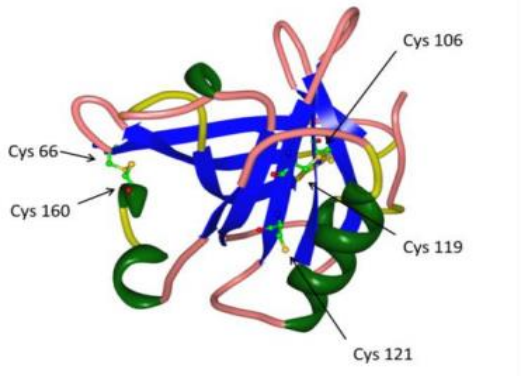

Figure 2. β-Lg structure with 5 cysteine residues and disulfide bonds (16).

Under physiological conditions, β-Lg is a dimer of two β-Lg protein subunits, with the balance to the monomer form, which allows the conversion of the dimer into native monomers (17, 18). The equilibrium towards monomer formation from the dimmer occurs at low β-Lg concentrations, at a low ionic strength, and at pH lower than 7 (19). Thus, β-Lg can associate and dissociate, depending on the pH of the environment. At the pH typical for milk, β-Lg is a dimmer. Due to a high electrostatic repulsion at pH 3.5, β-Lg reversibly dissociates into monomers (6). On the other hand, some authors have shown that β-Lg is a monomer at pH below 3.5 (7, 20) and also above 7.5 (21). The researchers (7) also reported that at pH 3.7-6.5, the β-Lg dimmer reversibly associates into an octamer.

It was established elsewhere that β-Lg can exist as tetrameric, octameric, and multimeric aggregates, in various environmental conditions.

β-Lg solutions form gels under different conditions when the native structure is destabilized to the extent that aggregation occurs. At the long-term thermal processing (prolonged heating) at a low pH and low ionic strength, the translucent "fine-stranded" gel is formed in which the protein molecules assemble into long and rigid fibers. The mechanisms of denaturation/aggregation of β-Lg protein were described by several researchers, for example (22). At neutral pH and room temperature, β-Lg is a stable noncovalent dimer. With an increase of temperature, the β-Lg dimmer dissociates into monomers that also undergo conformational changes (23).

Critical changes in the conformation of β-Lg consist in exposing hydrophobic residues and sulfhydryl groups on the β-Lg protein surface, making it available for intermolecular interactions (24, 25). A new β-Lg configuration was defined as the “molten globules” (22, 26). It is considered that the molten globules are intermediate states in the folding and unfolding globular proteins, but their characteristics are difficult because they are present in a transient state (27, 28).

Some proteins can form stable molten globule structures after destabilizing changes, such as removal of ligands, point mutation, mild-denaturant conditions, and the formation of non-native disulfide bonds throughout intramolecular rearrangements (28, 29).

At the initial stages of β-Lg denaturation/aggregation, caused by high temperatures, the non-native monomers are formed. The exact nature of these non-native forms has not been described so far. The formation of native monomers at high temperatures is due to the intermolecular disulfide bonds interaction Cys121 sulfhydryl/ Cys106-Cys119. At least, two monomers of β-Lg are present in heated solutions – one has a native sulfhydryl group of Cys121 exposed to solvent (Mcys121) and the other – disulfide non-native Cys106-Cys121 bonds and a free and exposed sulfhydryl nonnative group of Cys 119 (Mcys 119). While Mcys121 is reversible to the native form of β-Lg, Mcys119 has a stable conformation, which makes the return to the native form upon cooling impossible (18).

Peculiarities of whey proteins behavior under the action of high temperature and pH variations

Whey proteins are heat-labile. According to the research data (27), high temperatures decrease their stability in the following order: PP>α-La>β-Lg>BSA>Ig. Thermal denaturation of the whey proteins is a two-step process. In the first phase,

unfolding occurs, which may be reversible or irreversible and includes aggregation that usually follows the irreversible unfolding (9). Heat treatment causes significant deterioration of the protein structure, leading to the modification of physicochemical properties, including solubility, water-holding capacity, emulsifying, foaming and gelling (30). Unlike casein, whey proteins are completely denatured after 5 minutes of heating at a temperature over 90°C. In fact, whey protein denaturation begins at 65°C, especially during the heating of milk at temperatures over 80°C (31). It was also demonstrated elsewhere that heating at 85°C is critical for whey protein denaturation (32). The degree of protein denaturation is determined by the degree of β-Lg denaturation, since it makes up 50% of total whey proteins (30, 33).

Influence of high temperatures and pH on the behavior of β-Lg

As reported elsewhere, thermal denaturation of β-Lg occurs in two stages (12, 33-35, 36). In the first stage, dimmers dissociate and form four monomers, and then they interact via sulfhydryl groups and form small aggregates. Aggregate formation takes place at about 70°C (12, 33, 36), whereas the maximum speed is reached at 80-85°C (29, 37).

In the second stage (called non-specific), small aggregates interact through non-specific binding and high molecular weight (MW) aggregates are formed. According to some researches (34, 35), this stage occurs at temperatures higher than those of the first stage.

Such factors as pH, concentration of salts, sugar, and proteins have a significant influence on the thermal behavior of β-Lg. Some authors established the pH-dependent thermal denaturation of β-Lg (7, 9, 34, 35, 38).

β-Lg is most sensitive at pH 9.0, when denaturation is triggered at 43°C and destruction of the secondary structure is induced at 51°C. Thermal denaturation occurs at pH 6.0 and at 78°C. Denaturation takes place at pH 5.5 and at 76°C (9).

The maximal heat stability of β-Lg was recorded at pH 3.0 (7).

Dynamic aggregation of proteins results from the intermolecular association of various forces and bonds. Since the isoelectric point of β-Lg is at pH 5.3, a decrease in the pH up to 5.5 will minimize the intermolecular electrostatic repulsion, thus increasing the molecular aggregation via Van der Waals forces and hydrophobic chemical bonds. Aggregates and polymers that are formed at low pH may be heterogeneous, thus contributing to light diffusion and turbidity of the solution.

The pH dependent aggregation of proteins apparently differs from the real protein denaturation. It was demonstrated elsewhere that in low ionic strength solutions, an increase in pH from 5.5 to 6.5, might decrease the temperature of denaturation from 80°C to 70°C. Although β-Lg denatures at pH≥6.0 rather than at pH<5.5, an increase of pH up to ≥6.0 will increase the protein-water interaction, favoring thereby protein solubility. This effect will contribute to the rejection of protein charges and will weaken the protein-protein interactions. It was found elsewhere that the solubility of whey protein decreases markedly during heating at pH values between 5.0 and 5.5, but not greater than 6.0.

The main purpose of this work was to study the extraction of β-lactoglobulins in protein-mineral concentrates (PMCs) during the electrophysical processing of different types of whey, using different processing regimes, as well as pH and temperature variations.

MATERIAL AND METHODS

Three types of initial whey (IW) products were used during the experiments on electroactivation, which were provided by the "JLC" Joint Stock Company, Chisinau, R. Moldova, including the following: granulated cottage cheese „Grăuncior" – 1; "Cottage cheese", 2% fat content – 2; and „Curd product", 18% fat content – 3.

For convenience the, types of processed whey will be named according to their initial proteins content:

1 – whey with high proteins content (WHPC);
2 – whey with medium proteins content (WMPC);
3 – whey with low proteins content (WLPC).

The isolation of the casein powder does not influence the degree of proteins isolation; however, in this case, a lower quantity of proteins is isolated from each type of whey.

The quantitative modifications of the initial protein content before and after the isolation of

casein powder are shown in Figure 3.

In all the experiments, the electrophysical processing of whey was performed within the membrane electrolyzer EDP-2 (with a short distance between the electrodes and V/S=1.4), at a stationary regime and at a current density of 10 and 20 mA/cm^2, which remained constant during processing, and in a stationary regime of discharge of the working liquid (different types of whey discharged in the cathode cell) and of the secondary liquid (2% $CaCl_2$ solutions discharged in the anode cell).

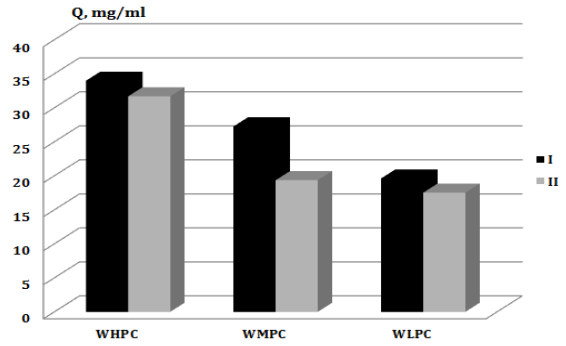

Figure 3. Initial protein content of 3 types of whey: I - IW without isolation of casein powder; II - IW with isolation of casein powder.

The PMCs at certain treatment periods were collected, whereas the results of experiments were reported by the authors earlier (39).

The quality of the IW and of the products obtained after electroactivation was determined according to the following physico-chemical and biochemical parameters:

- The pH of whey and the oxidation-reduction potential (ORP) were determined using a pH meter 766 (Knik, Germany).
- The temperature was determined in two phases: liquid and foaming. The chemical thermometer in the cathode cell was used in the liquid phase, while the pH-meter 766, Calimatic (Knick) was used in the foaming phase.

Protein content. The degree of protein recovery in the PMCs was determined by calculating the difference between the protein content in the IW and the remained deproteinized whey (DW), as shown in formula 1, separated in the field of mass forces by centrifugation at 1500 G via the Warburg method with the SF-56 spectrophotometer (40):

$$Q = Q_{ZI} - Q_{ZD}, \% \quad (1),$$

whereas:

Q – the protein content in the PMCs;
Q_{ZI} – the protein content in the IW;
Q_{ZD} – the protein content in the DW.

Protein fractions isolated from whey into the PMCs were analyzed with different concentrations of polyacrylamide gel via electrophoretic methods and three types of buffer solutions:

- **Buffer solution (I):** phosphate-citrate buffer (Me Ilvane) 0.5 M NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA) (0.04% NaN_3) pH 5.6, used in the isolation of proteins from the PMCs in the studied whey via the electrolyzer EDP-2, during the processing of WHPC and WMPC.
- **Buffer solution (II):** 0.05 M Tris-HCl buffer 0.5 M NaCl, 0.5 mM EDTA (0.04% NaN_3), pH 8.0, used in the isolation of proteins from the PMCs in the studied whey via the electrolyzer EDP-2, during the processing of WHPC and WMPC.
- **Buffer solution (III)**: 0.025M Tris-glycine buffer pH 8.47 used in the isolation of proteins from the PMCs in the studied whey via the electrolyzer EDP-2, during the processing of WHPC and WMPC.

The amount of soluble protein concentrates (Q_s, %) obtained from the above mentioned two types of whey, extracted with various types of buffer solutions is different, showing a higher solubility amount in buffer solution (II)-0.05 M Tris-HCI 0.5 M NaCI, 0.5 mM EDTA (0.04% NaN_3), pH 8.0 (fig. 4).

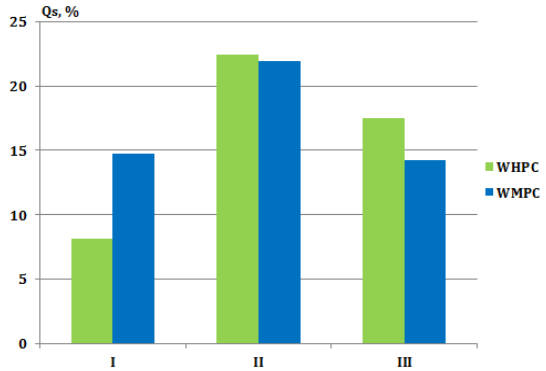

Figure 4. Content of soluble protein fractions (Q_s, %) of WHPC and WMPC recovered with buffer solutions (I-III).

The total content of soluble proteins (Q_s, %) recovered from the PMCs in the buffer solutions

mentioned above was determined by the micro-biuret method based on the interaction of peptide bonds with Cu^{2+} in a strongly alkaline medium, which results in the formation of a colored complex (41). The method is considered to be rapid, strict, and specific for protein estimation by using an alkaline copper sulfate reagent at the concentration of protein in the solution of 0.02 to 0.5 mg/mL. The sensitivity of this method is 0.003 mg/mL.

Reagents:

I - 0.28% $CuSO_4 \cdot 5H_2O$ solution in 30% NaOH

In order to avoid precipitation of $Cu(OH)_2$, when preparing the reagent, the solution of $CuSO_4 \cdot 5H_2O$ (1.4 mL of aqueous solution with 0.28g of $CuSO_4 \cdot 5H_2O$) was added dropwise in the alkaline solution (in 100 mL of 30% NaOH), and then vigorously stirred with a magnetic agitator.

II - 30% NaOH solution.

Procedure:

$A_{c.}$ – (control) 1 mL of reagent I is added to 2 mL of distilled H_2O.
A – 1 mL of reagent I is added to 2 mL of the investigated protein solution.
$B_{c.}$ – (control) 1 mL of reagent II is added to 2 mL of distilled H_2O.
B – 1 mL of reagent II is added to 2 mL of the investigated protein solution.

The prepared solutions were carefully stirred, kept for 15 minutes and then the optical density/absorption (A_{310nm}/E_{310nm}) in 1cm quartz cells at 310 nm on the spectrophotometer SF-56 was measured: A against A_c and B against B_c. The color of the prepared solution is maintained for 2.5 hours.

Further calculations were carried out on the difference of optical densities/absorptions, using formula 2:

$$\Delta A_{310nm} = A - B \quad (2)$$

The protein concentration is calculated on the previously made calibration curve (fig. 5). The calibration curves constructed for the aqueous solutions of BSA (from Serva) that were prepared by diluting the initial solution containing 1 mg/mL BSA (0.1% p-p).

The protein content via the micro-biuret assay was calculated as given below, using formula 3:

$$Y\ (mg/mL) = 0.77914 * X(\Delta A_{310nm}) + 0.00322 \quad (3),$$

whereas,

E – the optical density registered at the wavelength of 750 nm;
C – the standard protein concentration ABS, µg/mL;
R^2 – the approximate degree (R^2= 0.9999).

The approximation error is 1- R^2.

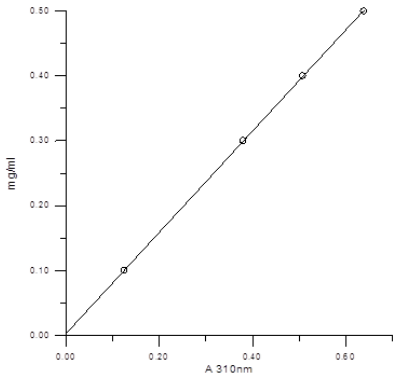

Figure 5. Calibration curve to determine soluble protein content isolated from PMCs via micro-biuret assay- BSA with concentration of 0.1-0.6 mg/mL.

Soluble proteins transferred in the buffer solution (protein buffer) were analyzed via electrophoresis with the sodium dodecyl sulfate (SDS-PAGE) and the 15% non-denaturing polyacrylamide gel (PAAG), according to (42).

The obtained results were scanned through the HP Scanget 3800 with the software Microsoft Photo Editor and analyzed with the Phoretix 1D Advans in order to determine the quantity of the major fractions in the PMC.

The recovery of β-Lg in the PMCs at electroactivation of different types of whey with different initial protein content was studied in the following configurations:

Configuration 1.
Electroactivation of WHPC at j=10 mA/cm², by collecting of whey in the form of foam at every 10 minutes (10-30 min – processing time) and CC is the content of the cathode cell, which represents the liquid phase.

Configuration 2.
Electroactivation of WHPC at j=20 mA/cm², by collecting of whey in the form of foam at every 5 minutes (5-15 min – processing time) and CC is the content of the cathode cell, which represents the liquid phase.

Configuration 3.
Electroactivation of WHPC at j=20 mA/cm², by collecting of whey in the form of foam at every

10 minutes (10-20 min – processing time) and CC is the content of the cathode cell, which represents the liquid phase.

Configuration 4.
Electroactivation of WMPC at j=10 mA/cm², by collecting of whey in the form of foam at every 10 minutes (10-30 min – processing time) and CC is the content of the cathode cell, which represents the liquid phase.

Configuration 5.
Electroactivation of WMPC at j=20 mA/cm², by collecting of whey in the form of foam at every 10 minutes (10-20 min – processing time) and CC is the content of the cathode cell, which represents the liquid phase.

Configuration 6.
Electroactivation of WLPC at j=10 mA/cm², by collecting of whey in the form of foam at every 10 minutes (10-30 min – processing time) and CC is the content of the cathode cell, which represents the liquid phase.

Configuration 7.
Electroactivation of WLPC at j=20 mA/cm², by collecting of whey in the form of foam at every 5 minutes (5-20 min – processing time), and CC is the content of the cathode cell, which represents the liquid phase.

RESULTS

Extraction of whey proteins and obtaining PMCs of a high value by electroactivation, as well as avoiding the direct usage of chemicals is an advantageous process based on modern principles, which ensures the finite cycle of the simultaneous processing of whey sugars (isomerization of lactose into lactulose) by separating them from the DW (fig. 6), (43).

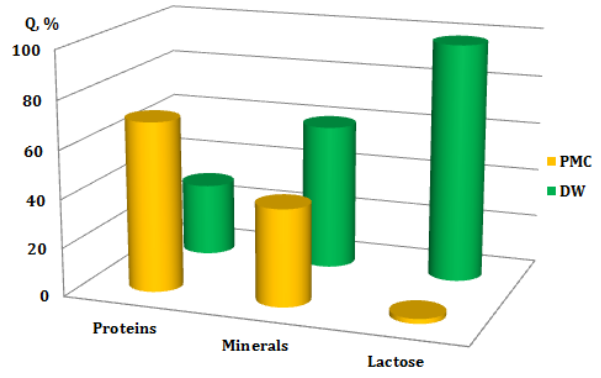

Figure 6. Electro-fractionation of DW by electroactivation.

The PMCs were investigated by electrophoretic techniques and processed via a membrane electrolizer EDP-2 at different current densities, by using different regimes of flow in different whey types, isolated with aforementioned buffer solutions.

Traditionally, these fractions make up four groups:

- high weight proteins (HWP), in which 2-5 fractions show various MW 54-249 kDa, containing the BSA with MW 66 kDa, lactoperoxidase with MW 70 kDa and lactoferine with MW 80 kDa, as well as protein complexes with MW of about 200-249 kDa;
- caseins (CSNs), in which 2-3 fractions: α-CSN, β CSN, and κ-CSN were identified, with MW 37, 33, and 46 kDa, respectively;
- β-Lg with a MW 18.4 kDa whose isolation is significant in all the collected samples;
- α-La with a MW 14.2 kDa that were isolated almost uniformly during the entire processing.

The electroactivation of WHPC, WMPC, and WLPC under all conditions mentioned above, and the identification of the content of soluble protein fractions in the 0.05 M Tris-HCl buffer 0.5 M NaCl, 0.5 mM EDTA (0.04% NaN_3), at pH 8.0, by using electrophoresis SDS-PAGE 15%, after major fractions mentioned above, demonstrated variations of the protein content.

The recovery of whey proteins in the PMCs (Q, %) from the IW varied depending on the solid content of each type of whey, on the processing regime (different current densities, the amount of the processed IW), on various electrical, thermal and physico-chemical parameters, and on the duration of treatment.

Whey proteins were most intensively recovered from WHPC, which had the highest initial protein content (the degree of recovery of whey proteins exceeds 60% at j=20 mA/cm² and 10 min of treatment), then followed by WMPC, showing an over 50% protein recovery in the PMCs, and WLPC, containing more than 50% whey proteins recovered in the PMCs (fig. 7, 8).

The major protein fractions (HWP, CSN, β-Lg, α-La) recovered in the PMCs by electroactivation of three types of whey were determined under

all the aforementioned conditions, using the electrophoretic analysis with SDS-PAGE 15%. The soluble proteins (Q_s, %) were studied in buffer solution (II) at pH 8.0, which allowed a more intense extraction compared to the use of buffer solution (I) with pH 5.6.

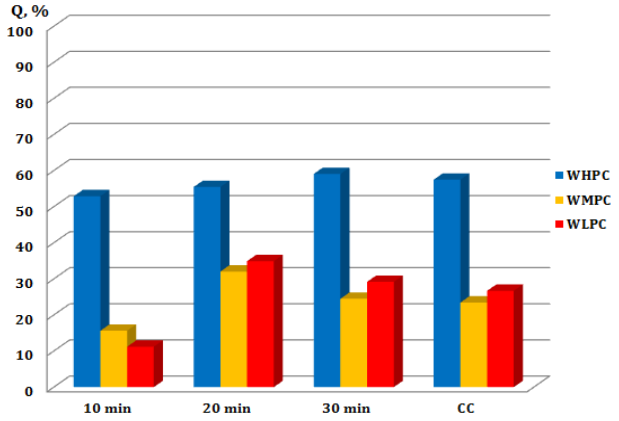

Figure 7. Recovery of whey proteins in PMCs, at processing of 3 types of whey at j=10 mA/cm^2.

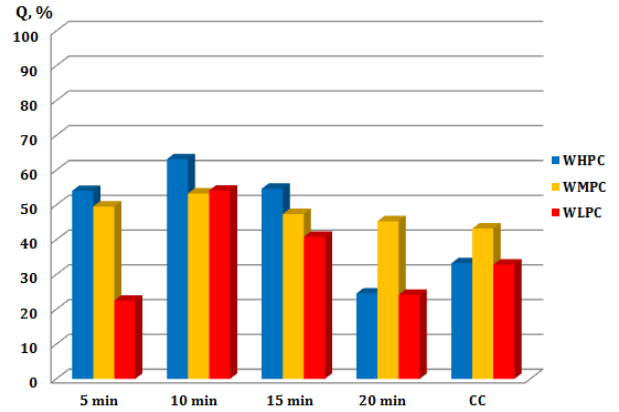

Figure 8. Recovery of whey proteins in PMCs, at processing of 3 types of whey at j=20 mA/cm^2.

Configuration 1.

The total content of soluble proteins (Q_s, %) recovered with buffer (II) pH 8.0, of the PMCs extracted from WHPC at j=10 mA/cm^2 varied depending on the processing time, however being almost twice lower than the total protein content recovered in the PMCs. This fact was due to the tangled electro-bio-chemical processes occurring in the formation of protein-mineral compounds and that could not be extracted with this type of buffer solution (fig. 9).

Electrophoretic analysis with SDS-PAGE 15% of soluble proteins under the mentioned conditions demonstrated a non-uniform extraction of major protein fractions (HWP, CSN, β-Lg, α-La), conditioned by both high protein content of the IW and total solid contents, as well as due to the electrohydrodynamic and electro-biochemical processes, including the ionic flotation, activated by electrochemical interaction (fig. 10, 11).

The whey studied in Configuration 1 allowed extracting the following β-Lg: 46.58% at 10 min of processing, 31.36% at 20 min and 42.89% at 30 min; whereas in the liquid phase – in 37.74%. β-Lg extraction also depends on various pH and temperature values.

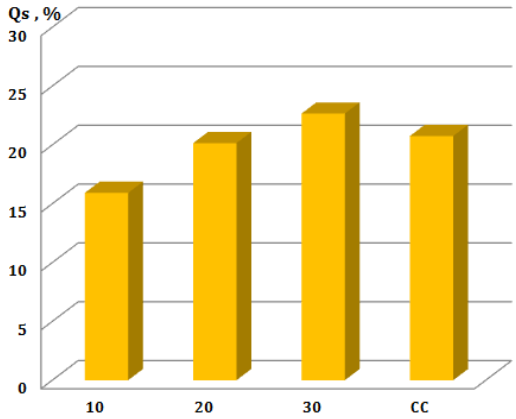

Figure 9. Variations of content (Q_s, %) of total soluble proteins recovered with buffer solution (II), pH 8.0, by electroactivation of WHPC at j=10 mA/cm^2, being processed for over 10-30 min.

On electroactivation, the protein molecules exhibited an oscillating electric field due to their amphoteric properties. The temperature gradient inside the cathode cell was non-uniform, increasing from 19 to 24°C, however, the temperature at the cathode surface may be higher than inside the cell, causing protein denaturation.

High temperatures were not registered in this configuration, thus excluding the thermal denaturation of β-Lg. Such factors as pH, concentration of minerals, carbohydrates, and proteins have a significant impact on the thermal behavior of β-Lg.

The pH value in the researched configuration increased on electroactivation from 4.53 to 9.20 in the first 10 minutes. The isoelectric point of β-Lg was – pH 5.3, causing a partial sedimentation of β-Lg in PMC.

It was mentioned elsewhere that β-Lg is the most sensitive at pH 9.0 and at temperature 43°C, which is the starting point for denaturation,

whereas at 51°C, the destruction of the secondary structure occurs (9).

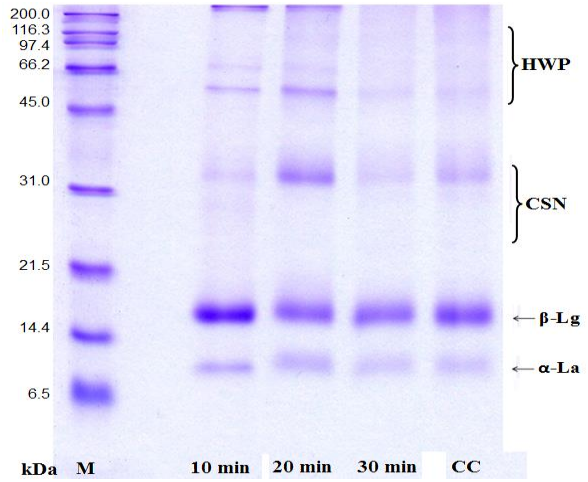

Figure 10. SDS-PAGE 15% of soluble protein concentrates of WHPC isolated with buffer solution (II), pH 8.0, by electroactivation at j=10 mA/cm², with marker M; over a 10-30 min processing time.

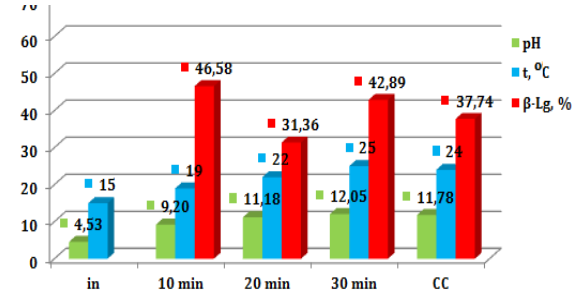

Figure 11. Variations of pH values, temperature and soluble protein fraction content (β-Lg, %) of WHPC, isolated with buffer solution (II), at pH 8.0, analyzed with SDS-PAGE 15%, by electroactivation at j=10 mA/cm²; over a 10-30 min processing time.

The aggregation of whey proteins involves the interaction of free-SH groups with the S-S bonds of cysteine-containing proteins such as β-Lg, k-CSN, α-La, and BSA through the – SH/S-S interactions (44).

These interactions between proteins lead to an irreversible aggregation of proteins into protein complexes of different molecular sizes, which depend on the heating conditions and the protein composition.

On electroactivation, it is assumed that β-Lg unfolding occurs and the hidden – SH groups become available on the surface of the molecule under these conditions, possibly as in the model described in (45). Since α-La and BSA are also present in whey, the reaction scheme could be extended by an extrapropagation step to accommodate the reaction between β-Lg and α-La or BSA (45).

A considerable increase of the pH values by the treatment causes the activation of the – SH/S-S groups and the extraction of β-Lg in the PMCs.

Configuration 2.

The total content of soluble proteins (Q_s, %) of the PMCs obtained from WHPC, recovered with buffer solution (II), at pH 8.0, being electroactivated at j=20 mA/cm², followed by the collection of PMCs at every 5 minutes, allowed a more detailed study of the extraction of protein fractions in the PMCs, by reducing the registration step.

The amount of soluble proteins varied from 20 to 25%, which confirmed the formation of protein compounds with a high MW, which could not be solved with this type of buffer solution (fig. 12).

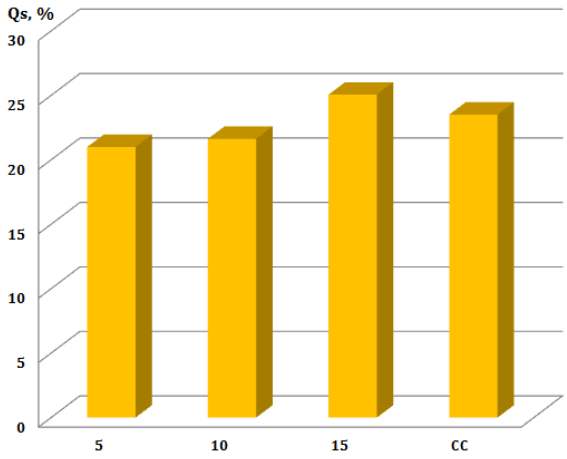

Figure 12. Variations of content (Q_s, %) of total soluble proteins recovered with buffer solution (II), pH 8.0, by electroactivation of WHPC, at j=20 mA/cm², and over a 5-15 min processing time.

An electrophoretic analysis with SDS-PAGE 15% of the content of protein fractions extracted from the PMCs with buffer solution (II), pH 8.0, by electroactivation of WHPC at j=20 mA/cm², at every 5 minutes of treatment, allowed the registration of the extraction of β-Lg fractions, which made up about 31.36% in the first 5 minutes of processing and 31.92% over 10 minutes of processing, whereas the total extraction of β-Lg made up 63.28%, compared to the results in

Configuration 1, where the total extraction of β-Lg was 46.58% over 10 min which confirms the "unfold-ding" of β-Lg, according to the models previously described (fig. 13, 14).

Both the temperature and pH values increased faster, leading to a more abundant total extraction of β-Lg over the first 10 minutes.

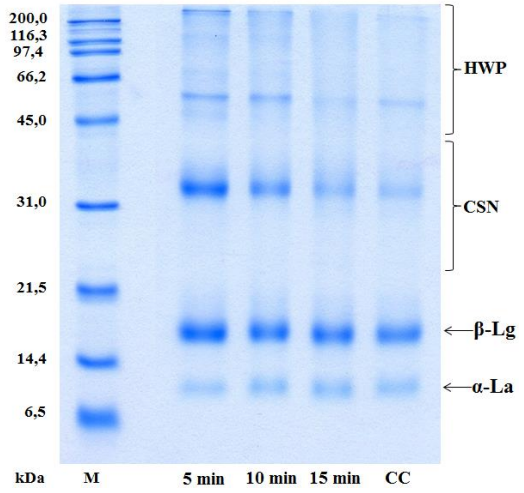

Figure 13. SDS-PAGE 15% of soluble protein concentrates isolated with buffer solution (II), at pH 8.0, by electroactivation of WHPC, at j=20 mA/cm², with marker M, over a 5-15 min processing time.

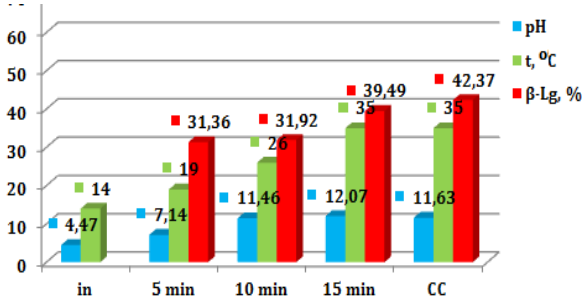

Figure 14. Variations of pH values, temperature and soluble protein fraction content (β-Lg, %) of WHPC, isolated with buffer solution (II), at pH 8.0, assayed by SDS-PAGE 15%, by electroactivtion at j=20 mA/cm², over a 5-15 min processing time.

Configuration 3.

The increased PMCs collection over 10 minutes allowed increasing the extraction of protein fractions in the PMCs.
The increasing electric current density, allowed increasing both the extraction of protein fractions in the PMCs, as well as the specific energy consumption per unit of processed volume.

The total content of soluble proteins (Q_s,%) extracted with buffer solution (II), pH 8.0, from the PMCs obtained from WHPC, electroactivated at j=20 mA/cm², PMCs being collected at every 10 minutes does not exceed 25%, which was considerably lower than the total protein content extracted in the PMCs (54-62%, see fig. 8), (fig. 15).

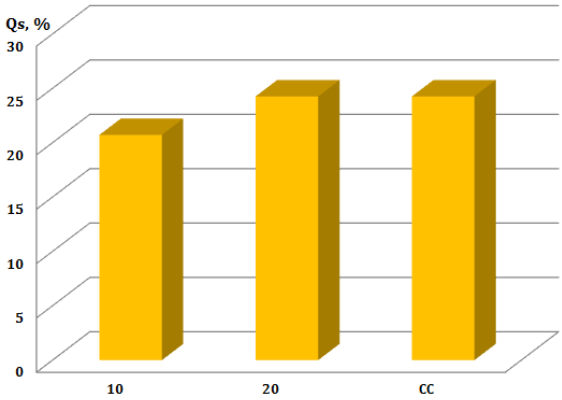

Figure 15. Variations of content (Q_s, %) of total soluble proteins recovered with buffer solution (II), pH 8.0, by electroactivation of WHPC at j=20 mA/cm², over 10-20 min processing time.

The electrophoretic analysis with SDS-PAGE 15% of the content of protein fractions of WHPC extracted from PMC with buffer solution (II), at pH 8.0, by electroactivation at j=20 mA/cm², at every 10 minutes of treatment, allowed the registration of an increased extraction of protein fractions in the PMCs, particularly of the amount of β-Lg is 54% over the first 10 minutes, however it showed a decreasing character during the processing.

Thus, the energy consumption was twice higher than while processing this type of whey at a j=10 mA/cm² current density, followed by an intensive formation of protein compounds with a high MW, which did not allow, however, their extraction with the mentioned buffer solution, as well as the electrophoretic assay with SDS-PAGE 15% (fig. 16, 17).

The temperature increased more intensely, compared to Configurations 1 and 2, reaching up to 47-48°C. The pH with intensely alkaline values caused the unfolding of proteins and the activation of the – SH/S-S groups, which facilitated the extraction of β-Lg, according to the described models, which also acted in the case of electroactivation.

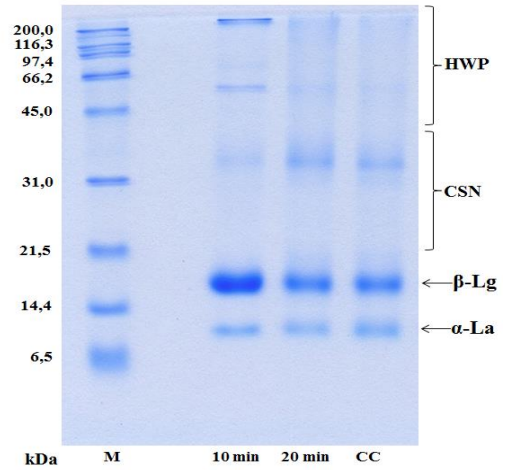

Figure 16. SDS-PAGE 15% of soluble protein concentrates isolated with buffer solution (II), pH 8.0, by electroactivation of WHPC at j=20 mA/cm², with marker M, over 10-20 min processing time.

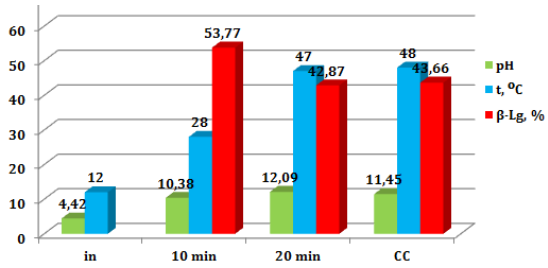

Figure 17. Variations of pH values, temperature and soluble protein fraction content (β-Lg, %) of WHPC, isolated with buffer solution (II), pH 8.0, assayed by SDS-PAGE 15%, by electroactivation at j=20 mA/cm², over 10-20 min processing time.

Configuration 4.

The whey electro-fractionation and PMCs formation with predetermined protein content is more obvious by electroactivating the WMPC.

The total content of soluble proteins (Q_s, %) extracted with buffer solution II, at pH 8.0, from the PMCs obtained by electroactivating WMPC at j=10 mA/cm² and the PMCs collection at 10 min intervals did not exceed 20%, which was considerably lower than the total protein content extracted in the PMCs (see fig. 7), (fig. 18).

The electrophoretic analysis with SDS-PAGE 15% of the content of protein fractions of WMPC, extracted from the PMC with buffer solution (II), pH 8.0, at j=10 mA/cm² and over a 10 min interval of treatment, allowed registering an increased rate of extracted protein fractions in the PMCs, particularly an increased amount of β-Lg (approximately 70%) was registered from the first minutes of processing, which however showed a decreasing tendency towards the end of the process (fig.19, 20).

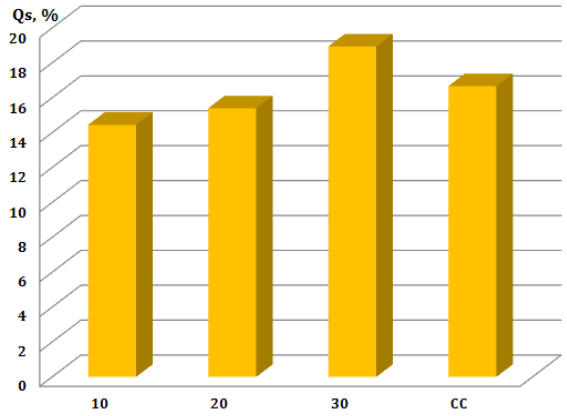

Figure 18. Variations of content (Q_s, %) of total soluble proteins recovered with buffer solution (II), pH 8.0, by electroactivation of WMPC at j=10 mA/cm², over 10-30 min processing time.

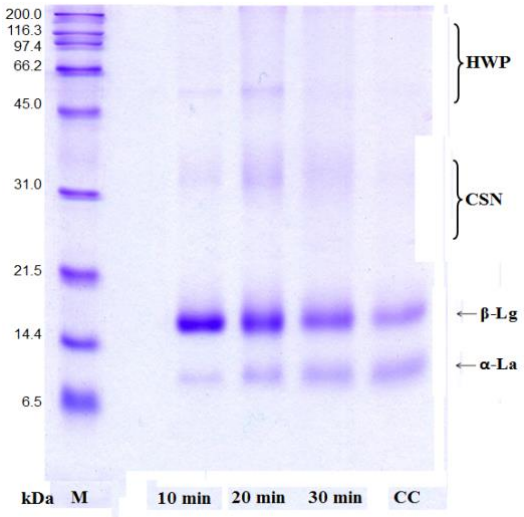

Figure 19. SDS-PAGE 15% of soluble protein concentrates isolated with buffer solution (II), pH 8.0, at electroactivation of WMPC at, j=10 mA/cm², with marker M, over 10-30 min processing time.

Configuration 5.

The total content of soluble proteins (Qs, %), extracted with buffer solution (II), at pH 8.0, of the PMCs obtained from WMPC, by the electroactivation at j=20 mA/cm² and the PMCs collection at every 10 minutes interval varied from 15% to 20%, which was considerably lower compared to the total protein content extracted in the PMCs (50-54% see fig. 8) (fig.21).

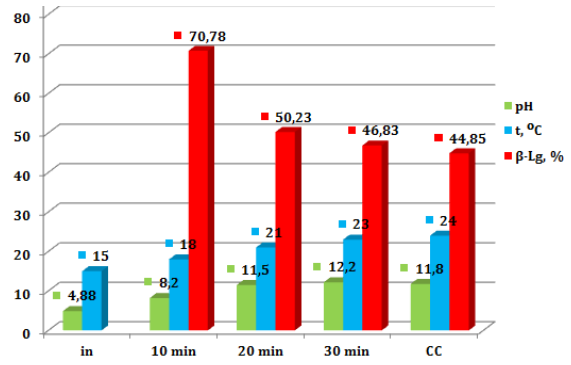

Figure 20. Variations of pH values, temperature and soluble protein fraction content (β-Lg, %) of WMPC, isolated with buffer solution (II), pH 8.0, assayed by SDS-PAGE 15%, by electroactivation at j=10 mA/cm², over 10-30 min processing time.

In this configuration, the pH values increased more slowly, and the amount of β-Lg (70.8%) was extracted at low alkaline values, which might be due to several mechanisms such as passing through the isoelectric point, the formation of dimers – oligomers of cystine, protein salination, etc.

At lower energy consumption, however, the β-Lg protein fraction did not exhibit a fast protein/protein interaction, which led to the formation of high-molecular polymers, thus allowing the extraction of a higher amount of β-Lg (46).

Thermal denaturation was excluded in this configuration and did not exceed 24°C. An abundant extraction in the first 10-20 mi-nutes of processing led to a reduced β-Lg content in the PMCs, obtained towards the end of the process.

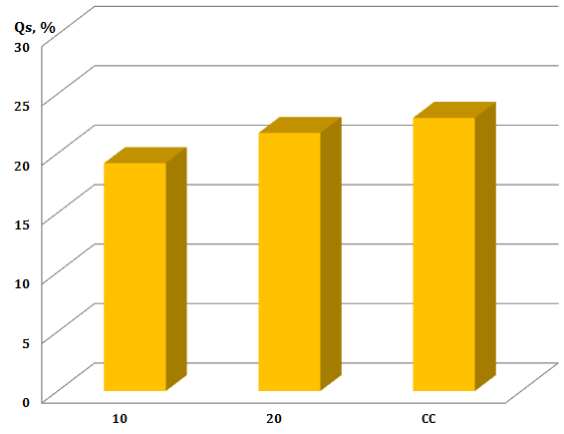

Figure 21. Variations of content (Q_s, %) of total soluble proteins recovered with buffer solution (II), pH 8.0, at electroactivation of WMPC at j=20 mA/cm², over 10-20 min processing time.

The SDS-PAGE 15% electrophoretis of the protein content, extracted from the PMCs with buffer solution (II), at pH 8.0, by electroactivation of WMPC at j=20 mA/cm² at every 10 min of treatment, allowed the extraction of β-Lg of 57-60%, which differed from that processed at j=10 mA/cm², under the same treatment conditions, the content of β-Lg being 71% (fig. 22, 23).

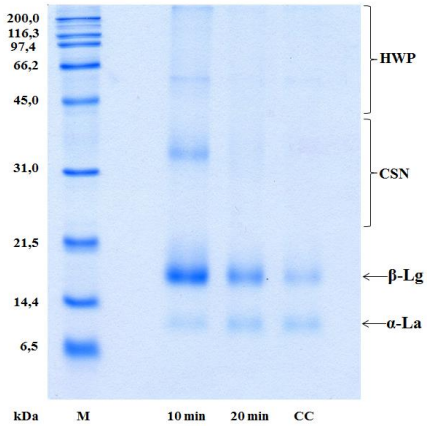

Figure 22. SDS-PAGE 15% of soluble protein concentrates isolated with buffer solution (II), pH 8.0, by electroactivation of WMPC at j=20 mA/cm², with marker M, over 10-20 min processing time.

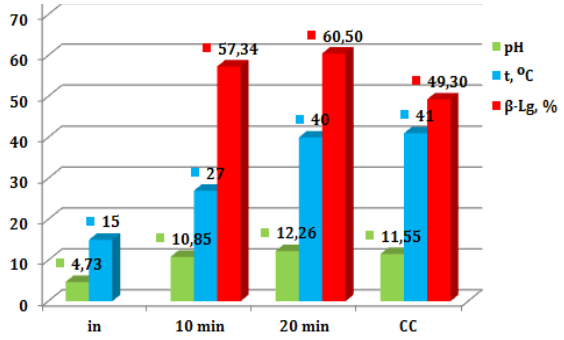

Figure 23. Variations of pH values, temperature and soluble protein fraction content (β-Lg, %) of WMPC, isolated with buffer solution (II), pH 8.0, by with SDS-PAGE 15% analysis, by electroactivation at j=20 mA/cm², over 10-20 min processing time.

Evidently, there was a very intense aggregation with other proteins, forming highly molecular protein compounds. The pH values increased considerably over the same processing time from pH 10.85 in the first 10 min to pH 12.26 at 20 min of treatment, which contributed to protein/protein aggregation (46). The high temperature did not cause thermal denaturation of the proteins, the maximum values being 40-41°C.

Configuration 6.

The electroactivation of WLPC at 10 mA/cm² obviously demonstrated different extraction degrees of whey protein fractions in the PMCs, which depended both on the processing regime, energy consumption, amount of processed whey, processing time, the pH variations, and on the type of the whey processed, which, in turn, depended on the primary processing of dairy products.

The total content of soluble proteins (Q_s, %), extracted with buffer (II), at pH 8.0 from the PMCs obtained from WLPC, electroactivated at j=10 mA/cm², varied non-uniformly during treatment, having a maximum value of 18% at the first 10 minutes. The amount of soluble proteins was lower than with the two types of whey described above, possibly, due to a lower protein content in the IW (fig. 24).

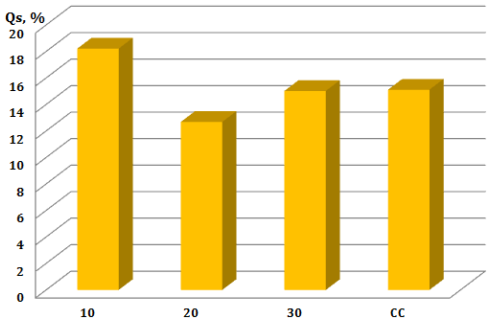

Figure 24. Variations of content (Q_s, %) of total soluble proteins recovered with buffer solution (II), pH 8.0, by electroactivation of WLPC at j=10 mA/cm², over 10-30 min processing time.

The SDS-PAGE 15% electrophoreris of the protein content of WLPC, extracted from the PMCs with buffer (II), at pH 8.0, by electroactivation at j=10 mA/cm², at every 10 minutes of treatment, registered an increased extraction degree of protein fractions in the PMCs.

The extraction of one of the major fractions of whey, β-Lg, was registered in the first 10 minutes of processing (51.35%), which takes place at neutral pH values (6.80), followed by a decrease of its content to intense alkaline values of pH 11.55-12.10. The processing temperature did not exceed the denaturation point (fig. 25, 26).

Configuration 7.

The total content of soluble proteins (Q_s, %) extracted with buffer solution (II), pH 8.0, of the PMCs obtained from WLPC, being electroactivated at j=20 mA/cm² and registered at every 5 min interval varied uniformly during treatment. The amount of soluble proteins was 20-25% (fig. 27).

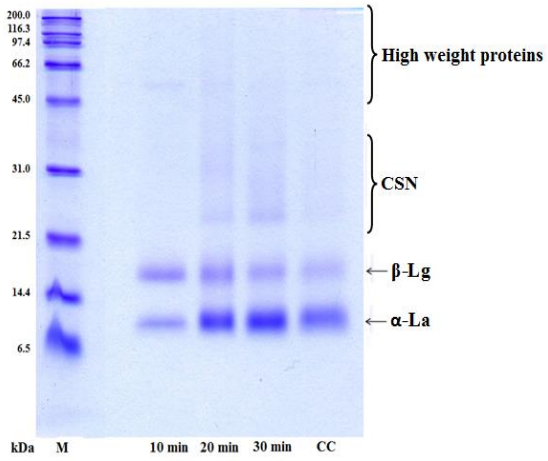

Figure 25. SDS-PAGE 15% of soluble protein concentrates isolated with buffer solution (II), pH 8.0, by electroactivation of WLPC at j=10 mA/cm², with marker M, over 10-30 min processing time.

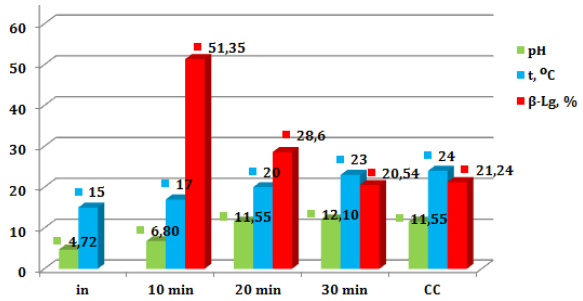

Figure 26. Variations of pH values, temperature and soluble protein fraction content (β-Lg, %) of WLPC, isolated with buffer solution (II), pH 8.0, by SDS-PAGE 15% analysis, by electroactivation at j=10 mA/cm², over 10-30 min processing time.

The SDS-PAGE 15% electrophoresis of protein content of WLPC, extracted from the PMCs with buffer solution (II), at pH 8.0, electroactivated at j=20 mA/cm² and at a 5 min interval of treatment, registered an increased recovery of protein fractions in the PMCs. An increased amount of β-Lg (66%), extracted in the PMCs from WLPC was recorded over the first 5 min of treatment, showing the highest value compared to those of the two types of whey previously studied (WHPC and WMPC) under similar processing conditions, which also decreased during processing (fig. 28, 29).

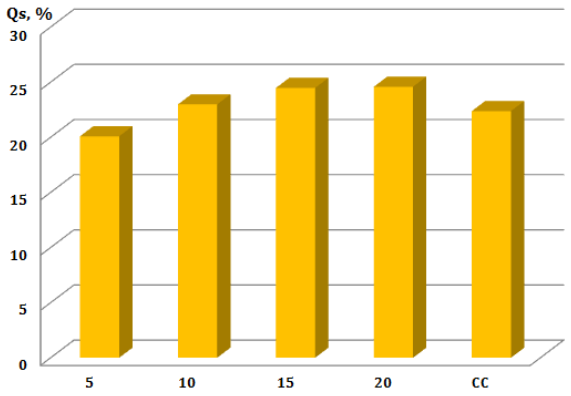

Figure 27. Variations of content (Q_s, %) of total soluble proteins recovered with buffer solution (II), pH 8.0, by electroactivation of WLPC at j=20 mA/cm² and over 5-20 min processing time.

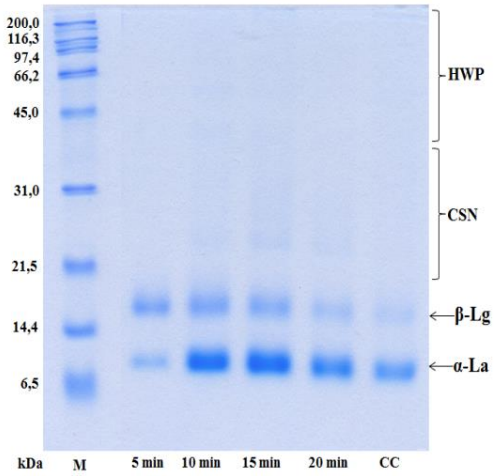

Figure 28. SDS-PAGE 15% of soluble protein concentrates isolated with buffer solution (II), pH 8.0, by electroactivation of WLPC at j=20 mA/cm², with marker M, over 5-20 min processing time.

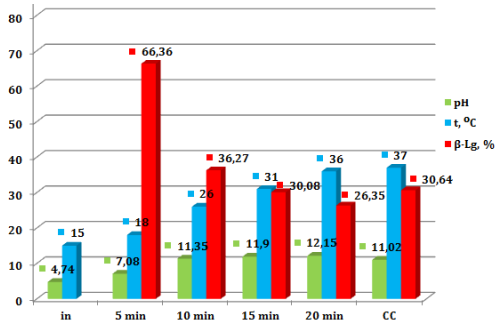

Figure 29. Variations of pH values, temperature and soluble protein fraction content (β-Lg, %) of WLPC, isolated with buffer solution (II), pH 8.0, analyzed by SDS-PAGE 15%, by electroactivation at j=20 mA/cm² and over 5-20 min processing time.

The maximum amount of β-Lg was extracted at neutral pH values, compared to Configuration 2, likely due to a higher initial content of β-Lg in this type of whey.

The processing temperature did not exceed the denaturation point. Moreover, the temperature-increasing rate was lower than in Configuration 2 due to a lower initial protein content (see fig. 8), which reduced the resistance of the processed medium, thus leading to a decrease of the Joule effect.

Whey electro-fractionation and PMCs formation with predetermined protein content was more clearly demonstrated on WLPC. The correct management of electroactivation by using different treatment regimens will allow the electro-fractionation of different types of dairy by-products.

DISCUSSIONS

Various and non-uniform isolation of protein fractions in the PMCs by electroactivation of the three types of whey was determined. First, depending on the properties of each fraction separately and then on their behavior resulting from the electrochemical action.

The explanation of the physical-chemical and biochemical properties of the whey proteins and the reasoning support of the formation of the PMCs requires a clear information on their structure. The phenomena and behavior of certain structural peculiarities of whey proteins might account for the action of some factors, such as temperature, pH, and ORP.

The whey PMCs formation depends on the electroactivation regime and the following parameters: electric current density, energy consumption per volume unit, variations of the pH and ORP values, temperature variations and Joule heat. Multiple inter- and intra-molecular processes occur under the action of the electric current on the solid shear of whey on about 6% and over 200 ingredients, which cause variations in the isolation of protein fractions in the PMCs

Whey proteins have a high solubility within a wide range of pH values owing to the composition of amino acids and the arrangement of their free radicals during the formation of tertiary structures. The pH increase is also compulsive due to processes generated by electroactivation.

It is due to the arrangement of hydrophilic radicals and of a large number of disulfide bonds on the surface of globular structures of whey proteins, which is similar to other research data. Furthermore, the globular structure provides resistance of those proteins to proteolysis.

The experimental data on the isolation of β-Lg viz. the most abundant whey protein, within the first 5-10 min, when the pH was kept within the neutral range (pH 7.0-8.0), at a low temperature registered in the volume of the CC, but which can be higher on the cathode surface, allowed concluding that reactive sulfhydryl groups (thiols) play an important role in the isolation of whey proteins by cysteine radicals.

Upon the oxidation of sulfhydryl groups of two cysteine radicals and the formation of covalent disulphide bonds (–S–S–) in proteins, a cystine is formed, which is a dimer of cysteine that jointly with hydrogen, ionic, and hydrofolic bons maintains the spatial structure of protein molecules. Similarly, intermolecular bridges are formed between cysteine radicals, dissociated both as a result of electroactivation and due to the increase inpH up to 8.3. The study results have proved that cysteine radicals participate in the PMCs formation of whey proteins, by blocking of sulfhydryl groups with Sodium iodoacetate. Introducing it into the initial whey reduces the isolation of proteins via the elimination of agregation of prot eins according to the mechanism described. The aggregation of whey proteins via various mechanisms and modes has been mostly presented earlier (47).

Sedimentation of proteins in their isoelectric point (pI) is one of the mechanisms that influences the extraction of β-Lg in PMCs. The variation of the active acidity in the cathode cell is not homogeneous, being conditioned both by its size/dimensions and by the initial whey flow at treatment, which increases the pI by certain protein fractions and obviously their sedimentation in pI during processing.

Isolation of whey proteins in the PMCs via the processing regimens depends on the action of several mechanisms of formation of protein compounds and on coagulation. An important role of Ca in the isolation of proteins has been confirmed by lowering its amount in deproteinised whey during processing and its presence in the mineral composition of the PMCs.

During electroactivation of whey, the concentration of ions in the electrolyzer zone might be several times higher (at the level of an order of magnitude) than in the initial solution, thus creating conditions for proteins salinity, which was indirectly confirmed by an intensive foaming in the cathode zone and by their later isolation supported by ionic flotation due to electroactivation. Protein salinization is one of the main mechanisms in the recovery of β-Lg in the PMCs.

The maximum amount of β-Lg recovered in the PMCs on electroactivation is 66% from WLPM and 71% from WMPC, which differed depending on the treatment regimes. Obviously, that the recovery of β-Lg is higher while extracting β-Lg from whey with a lower initially protein content.

The temperature registered in the cathode cell was not over 55-60°C, hence permitting PMCs formation without undergoing thermal denaturation.

CONCLUSIONS

The correct management of the electroactivation with various treatment regimens will allow the electro-fractionation of different types of dairy by-products.

1. The recovery of β-Lg in protein-mineral concentrates (PMCs) by electroactivation of whey depends on the electric current density, initial protein content, temperature, and pH values.

2. Electroactivation of whey with high initial protein content allows the extraction of a lower amount of β-Lg in PMCs due to intense electro-bio-chemical interactions, leading to the formation of protein-mineral compounds with a high molecular weight.

3. The recovery of β-Lg in PMCs, at the electroactivation of whey with medium and low initial protein content was more obvious, registering the maxima of 71% and 66%, respectively, due to a lower protein content in the initial whey, which allowed a more intense recovery.

4. The maximum amount of β-Lg recovered in PMCs was extracted within the first 5-10 minutes of treatment at low alkaline pH 7.00-8.00.

5. The temperature in the cathode cell was not over 55-60°C, hence permitting PMCs formation without undergoing thermal denaturation;

6. The main mechanisms that allow the recovery of β-Lg in PMCs are as following:

- Activation of the –SH/S-S groups and the extraction of β-Lg in the PMCs while increasing the pH values during treatment.
- Sedimentation of proteins in their isoelectric points.
- Protein salinization is the major mechanisms in the recovery of β-Lg in the PMCs.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest

ACKNOWLEDGMENT

This research was carried out within the STCU project 6011 and partially in the state program ANCD 20.80009.5007.06 (2020-2023).

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Date of receipt of the manuscript: 01/06/2020
Date of acceptance for publication: 19/12/2020

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OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

CASE PRESENTATION – STUDIU DE CAZ – PRESENTATION DE CAS CLINIQUE – ПРЕЗЕНТАЦИЯ СЛУЧАЕВ ИЗ КЛИНИЧЕСКОЙ ПРАКТИКИ

MEDICAL SCIENCES

OPEN ACCESS

CASE REPORT: ACUTE LIVER FAILURE INDUCED BY PARACETAMOL TOXICITY

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DOI: 10.38045/ohrm.2021.1.08 UDC: 616.36-008.64-036.11-02:615.212.099

Key words: *Paracetamol, overdose, N-acetyl-cysteine, hepatotoxicity, acute liver failure.*

Introduction

Paracetamol is the most commonly used drug across Western Europe and North America. There is no such trend of using Paracetamol in the Republic of Moldova, thus overdose cases are extremely rare. The mechanisms leading to overdose episodes might induce hepatotoxicity.

Clinical case

This present article reports a case of acute liver failure induced by Paracetamol overdose. A 46-year-old man presented to the Medpark International Hospital. He was administered Paracetamol 1000 mg, approximately every 4 hours for 2 days, and 1000 mg twice a day for another 2 days, after an intense physical exertion, followed by muscle fever with myalgia and low-grade fever (37.4 °C) The reason for asking the medical care were pronounced fatigue, nausea, loss of appetite, night sweats, and frequent urination.

Conclusion

Eventually, the case was successfully resolved due to N-acetyl-cysteine, administered according to the established treatment scheme, as well as the adjuvant therapy.

Cuvinte cheie:
Paracetamol, supradozaj, N-acetilcisteină, hepatotoxicitate, insuficiență hepatică acută.

INSUFICIENȚĂ HEPATICĂ ACUTĂ INDUSĂ DE SUPRADOZAJUL DE PARACETAMOL: CAZ CLINIC

Introducere

Paracetamolul este cel mai frecvent utilizat medicament în Europa de Vest și America de Nord. În Republica Moldova nu există, însă, această tendință și în consecință, cazuri de supradozaj înregistrate se atestă foarte rar. Mecanismele ce se derulează în timpul episoadelor de supradozaj induc hepatotoxicitate.

Cazul clinic

În acest articol relatăm un caz de insuficiență hepatică acută indusă de supradozajul cu Paracetamol. Un bărbat de 46 ani s-a adresat la Spitalul Internațional Medpark, care în urma unui efort fizic intens, ce a cauzat febră musculară cu mialgii și subfebrilitate (37,4°C), a administrat comprimate Paracetamol, 1000 mg aproximativ la fiecare 4 ore 2 zile, după care câte 1000 mg de 2 ori pe zi încă 2 zile. Ulterior au apărut simptome de fatigabilitate pronunțată, grețuri, inapetență, transpirații nocturne și micțiuni frecvente, acestea fiind motivul solicitării ajutorului medical.

Concluzii

Datorită medicației cu N-acetilcisteină, aplicată conform schemei de administrare instituite, cât și a tratamentului adjuvant, cazul respectiv a fost soluționat cu succes.

INTRODUCTION

Paracetamol (Acetaminophen) is an antipyretic and analgesic drug, used for mild to moderate pain, exhibiting a central action and being an active metabolite of phenacetin. Paracetamol was introduced onto the pharmaceutical market in 1950 and is currently the most widely used drug worldwide, used to treat pain and fever. Acetaminophen toxicity is defined as an excessive drug intake, greater than 4 g/24 h, which has become a common reason for medical referring to Emergency Healthcare Assistance across Western Europe.

CLINICAL CASE

A 46-year-old man referred to the doctor, complaining of pronounced fatigue, nausea, loss of appetite, night sweats, and frequent urination. He claimed that the symptoms occurred about a week ago, after an intense physical exertion, followed by muscle fever with myalgia and low-grade fever (37.4°C). He individually administered Paracetamol 1000mg, approximately every 4 hours for 2 days, afterwards 1000 mg twice a day for another 2 days. 24 hours passed since the last administration of Paracetamol. The objective examination did not reveal any particularities, except for the subicteric tint of the skin.

The complete blood count, urinalysis, urea and creatinine levels showed no pathological changes.

The laboratory biochemical findings revealed changes in liver functioning, suggesting hepatic cytolysis syndrome: Alanine aminotransferase (ALT) – 8009 U/L (normal range: 0.1-41 U/L), Aspartate aminotransferase (AST) – 3792 U/L (normal range: 0,1-41 U/L) (fig. 1); cholestasis: Gamma-glutamyl transferase (GGT) test – 210 U/L (normal range: 15-85 U/L) (fig. 2), total serum bilirubin level – 92 µmol/L (normal range: 3-17µmol/L), and conjugated bilirubin – 80 µmol/L (fig. 3). Moreover, the patient exhibited coagulopathy syndrome, confirmed by the quantitative D-Dimer assay: 7615 µg FEU/mL (norm <500µg FEU/mL) on the first day of admission and ranging values on the subsequent days of treatment. Prothrombin index made up 34.4% (70-130%) (fig. 4) and INR – 2.07. The albumin level showed the lowest value in the first days of treatment. Markers of viral hepatitis: Total anti-HCV, HBsAg, Total Anti-HBc, Total Anti-HDV were negative. The chest X-ray revealed no particular features. Magnetic resonance cholangiopancreatography (MRCP), performed to exclude gallstone disease and other etiologies, registered severe edema of the gallbladder wall (13 mmthick) and a vermicular lumen. A detached gallbladder and inability to visualize the intrahepatic bile ducts were reported. Periportal inflammation and edematous liver hilum. Early hepatomegaly. Were these MRI signs of acute hepatitis/acute liver failure?

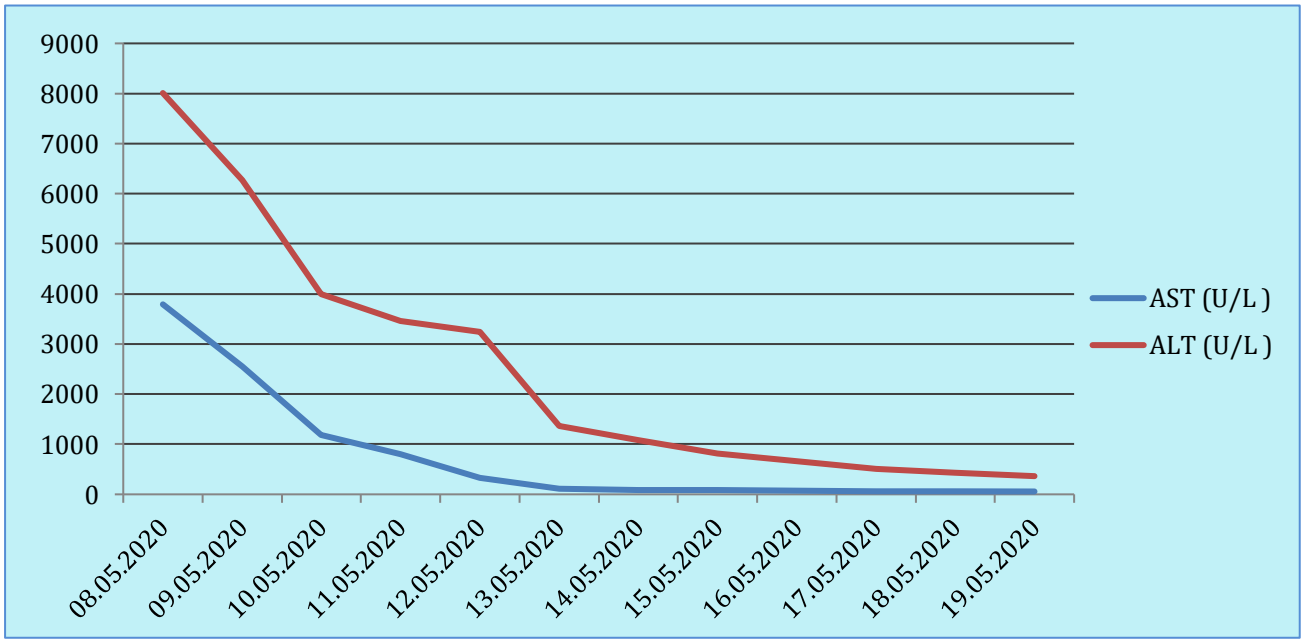

Figure 1. The dynamic changes of Alanine Aminotransferase and Aspartate Aminotransferase levels in patient with acute Paracetamol toxicity.

Severe acute liver injury was determined by elevated transaminases and INR=2.07 (>1.5) and jaundice (1). In this case, severity score for drug induced liver injury was moderate-severe (2). There were no clear signs of hepatic encephalopathy. The Glasgow Coma Scale didn't change.

The patient was given an emergency assistance within the Intensive Care Unit, by administering N-Acetyl-cysteine (NAC) at a loading dose of 150 mg/kg over the first 15 minutes, then 50 mg/kg – over the next 4 hours, and 100 mg/kg NAC – during the following 16 hours, thus, the total dose amounted for 300 mg/kg over 20 hours. On the third day of hospital stay, the treatment was combined with semi-pulse therapy with Methylprednisolone 500 mg for 3 days, which was further gradually reduced. Furthermore, on the 4th day of hospitalization, plasmapheresis and albumin transfusion, amino acids and anticoagulation with Enoxaparin, caused by the existing thrombotic risk (D-Dimer assay: 7615 µg FEU/mL) were carried out, showing a positive dynamic in patient's condition. The patient was discharged after 11 days of treatment with ALT – 371 U/L, AST – 57 U/L, GGTP – 233 U/L, the total serum bilirubin level being 50 µmol/L. The patient exhibited satisfactory overall condition and no adverse reactions were reported.

A follow-up treatment was recommended, by administering ademetionine and enterosorbents, as well as by maintaining the anticoagulation level by taking prophylactic doses of Enoxaparin for 1 month. Additionally, a dynamic monitoring of transaminases, bilirubin, GGTP levels, as well as a magnetic resonance cholangiography were performed.

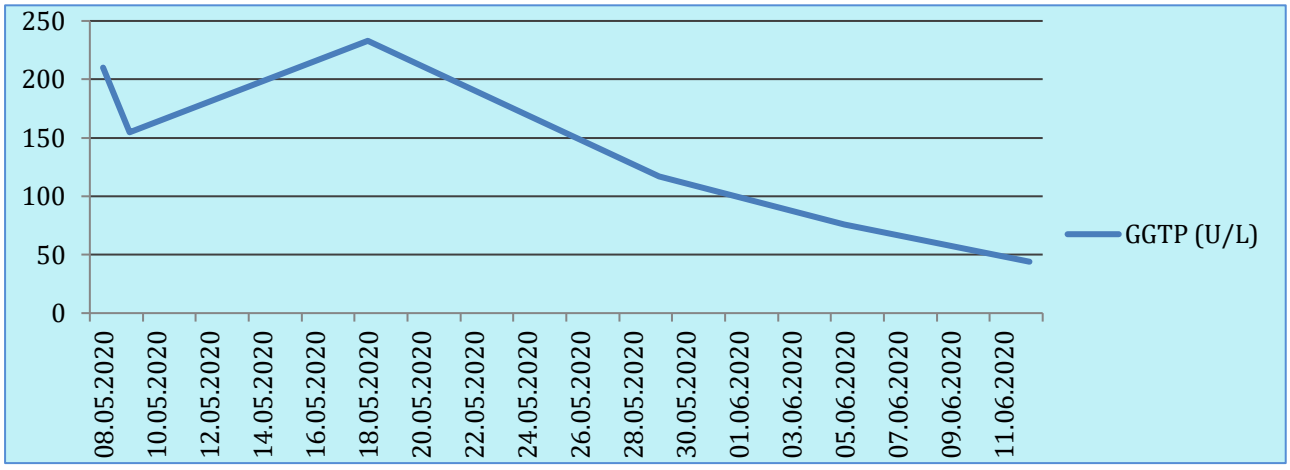

Figure 2. Dynamic changes of Gamma-glutamyltranpeptidaselevels in patient with acute toxicity.

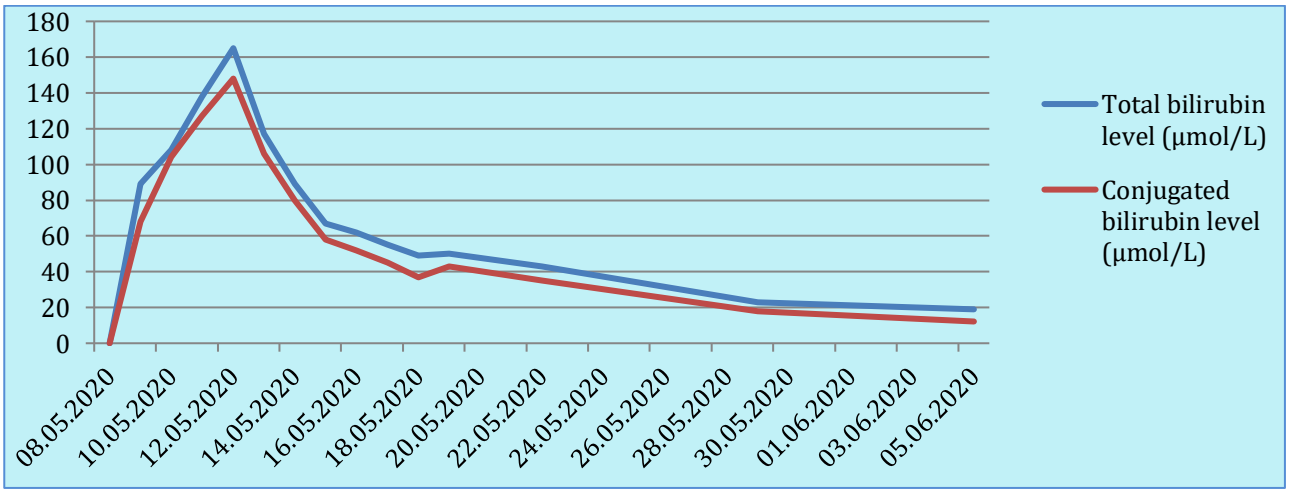

Figure 3. Dynamics of serum bilirubin levels in patient with acute Paracetamol toxicity.

Two weeks after discharge, the total serum bilirubin was 43 µmol/L, GGTP – 117 U/L, ALT – 343 U/L, AST – 76 U/L, whereas one month later since the disease onset, the biochemical assay revealed no signs of cholestasis and a low-level cytolytic syndrome (ALT – 59 U/L, AST – 32 U/L), without any differences from the laboratory reference ranges.

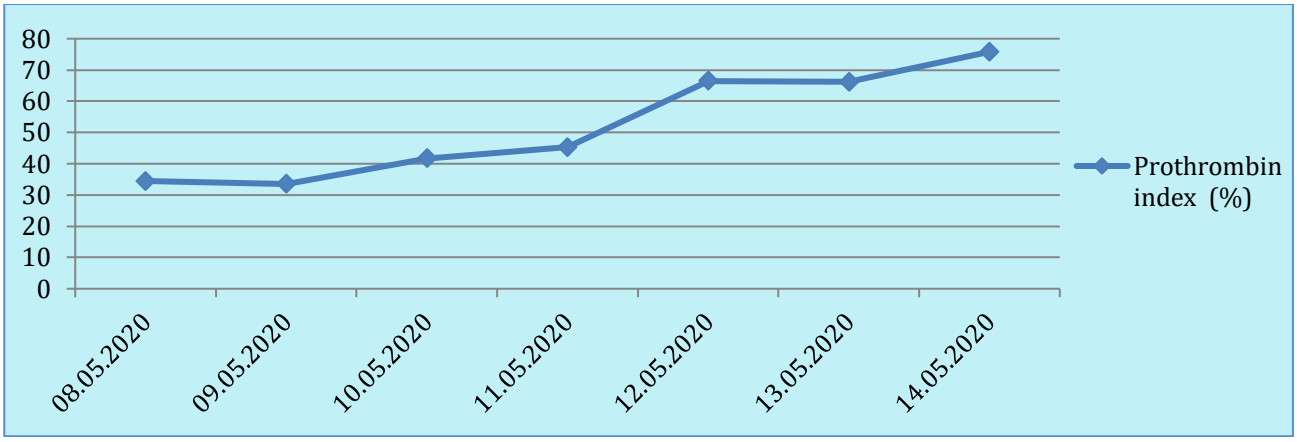

Figure 4. Dynamics of Prothrombin index throughout the treatment period.

DISCUSSIONS

There are approximately 100,000 referrals to the Emergency Department in the UK annually, caused by Paracetamol intoxication, resulting in about 150 deaths. The causes of overdose might be either due to unintentional use a wide range of drug availability containing acetaminophen, as well as due to a deliberate suicidal desire (3). In the United States, approximately 74-92% of Acetaminophen overdoses result from suicide cases, thus approximately 39% cases of acute liver failure are caused by Acetaminophen overdose (4, 5). However, Acetaminophen toxicity is not caused by only overdose. Sometimes, other reasons might occur, which lead to hepatotoxicity due to a definite therapeutic dose. Cases of Paracetamol toxicity have been reported in doses less than 4.0 g/24 hours in patients with Gilbert's syndrome, polymorphism in cytochrome P-450 (as well as other enzymes involved in hepatic metabolism), in malnourished patients, in people with chronic alcohol abuse, in elderly people, etc. (6). Paracetamol-induced acute hepatic failure cases quite rarely occur in the Republic of Moldova. After the drugs ingested per oral, it is absorbed very fast, reaching the highest plasma concentration in about one hour. The bioavailability is about 75%. When a healthy person administers a therapeutic dose of Paracetamol, it is conjugated directly into the liver to form sulfate and glucuronide derivatives. Thus, when administering a therapeutic dose, 55% of drug will be excreted as glucuronide, 30% as sulfate and 4% as metabolic oxidation by-products. The half-life (t½) is about 1.5-2.5 hours, however it might be prolonged in case of overdose (7, 8). The minimum toxic dose of Paracetamol is considered 10 g. The first symptoms appear within 4-12 hours after the first ingestion, featured by dyspeptic symptoms. Liver involvement occurs within 24-48 hours; however, symptoms of acute liver, kidney and heart failure might occur even over 4-6 days after ingestion (6).

The acetaminophen-induced hepatotoxicity mechanism leads to formation of toxic metabolite N-acetyl-para-benzo-quinone by cytochrome P-450, which induces oxidative stress, ATP depletion and mitochondrial dysfunction. This product is conjugated by hepatic glutathione and transformed into complexes that are excreted by the kidneys. This is a dosage-dependent mechanism. Another mechanism results in the production of peroxynitrite, which is a toxic free radical that induce oxidative stress via mitochondria dysfunction and DNA fragmentation. All these changes lead to an increased membrane permeability, vacuolation, caryolysis and, eventually, cellular apoptosis (2). NAC acts as a glutation donor, thus being an effective antidote. However, there are differences regarding the drug administration schemes. The recent studies state that NAC administration should be elective within the first 24 hours after ingestion of Acetaminophen (3, 9).

The homeostasis disorder between procoagulant and anticoagulant factors is mainly caused by liver failure. Moreover, the circulating endotoxins might also cause hypercoagulant effect, which might be also induced by acute liver failure (10). The specialized studies reported an increased heterogeneity of coagulation system abnormalities, ranging from a hypercoagulant to hypocoagulant status or the alternation of both. However, thrombosis is the most commonly reported complication. Prophylactic correction of coagulation is not recommended, however a reasonable monitoring and correction of coagulogram markers should be carried out (1, 7).

Both oral and parenteral administration of NAC are available. The disadvantage of oral administration is that a lower drug dose will be absorbed in case of vomiting episodes. The research studies, conducted since the 1970s, were aimed at identifying an effective antidote for Paracetamoltoxicity, by studying cysteine, methionine, and cysteamine, which have proven to have satisfactory treatment outcomes in small randomized trials. However, despite the effectiveness, there have been alsoreporteda series of side effects such as severe headaches, nausea and vomiting, thus leading to search for a new antidote. NAChas become the elective antidote, since it can be also administered intravenously and due to its rarely occurring side effects for the same therapeutic effect. The first 20-hour intravenous NAC regimen was based on the 4-hour Paracetamol half-life used by the British researchers. In the United States, another treatment scheme was developed on the prolonged half-life of Paracetamol, which can last for 12 hours in case of overdose. Despite minor changes in the administration regimen, it is still used effectively nowadays, except for some situations requiring abatement from the standard scheme. As for example in patients who continued to exhibit elevated plasma levels of Paracetamol or non-decreasing transaminases. These situations require an on-going NAC i.v. infusion until over 20-21 hours.

The length of the loading dose has been changed over time, ranging from 15 minutes (the traditional scheme) up to one hour to reduce the administration errors, which is still a controversial issue due to the adverse reactions. Other disputes have arisen regarding the reduction of NAC administration time to 12 hours, whether to switch from the so-called "three bag-regimen" to "two-bag regimen" scheme in order to minimize side effects. A number of randomized studies have been carried out in this regard, which resulted in positive treatment outcomes. The emergence of a great number of changed regimens confirm the low rate of adverse reactions, however showing a decreased therapeutic efficacy (11).

Corticosteroids are admitted in idiosyncratic drug-induced acute liver failure, when other treatments fail, showing clinical benefits (1, 2). In our clinical case, the use of Metilprednisolone was reasoned by the worsening of cholestatic and hepatodepressive syndrome on the third day.

The increasing number of drug abuse and toxicity cases worldwide, has determined the Food and Drug Administration (FDA) to establish the following criteria, in 2011: any drug containing Paracetamol, combined with other substances should not exceed the dose of 325 mg of Paracetamol per tablet. Thus, according to FDA decision, since January 2014, more than half of the pharmaceutical companies have limited the amount of Paracetamol in combined medications (3). However, drug availability and presence of acetaminophen in multiple combined pharmaceutical drugs of different brands poses a significant risk for over dosage. Furthermore, as a result of hepatotoxicity-related cases reported when using common therapeutic doses, current medicine tends to focus on precision medicine. It aims to study the human genome by genetic sequencing (6) in order to obtain data on the possibility of safe administration of Acetaminophen, if this medication is required to be administered.

CONCLUSIONS

The multitude of therapeutic regimes indicates a lack of a "gold standard", but medical practice shows that each patient require a customized treatment regimen due to a series of factors such as genetic, demographic and biological, as well as due to the amount of Paracetamol administered.

CONFLICT OF INTERESTS

The authors declare no conflict of financial or non-financial interests.

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Date of receipt of the manuscript: 16/07/2020
Date of acceptance for publication: 20/12/2020

OH&RM ONE HEALTH & RISK MANAGEMENT

VOL. 2, ISSUE 1
2021

EXPERTS' OPINIONS – OPINII ALE EXPERȚILOR – AVIS DES EXPERTS – МНЕНИЯ ЭКСПЕРТОВ

LIFE SCIENCES PROFESSIONAL RESPONSIBILITY IN AN AGE OF DUAL-USE RESEARCH

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INTRODUCTION

Biotechnology is progressing at an unprecedented pace promising to bring tremendous benefits by responding to health, socio-economic, and environmental challenges. At the same time, cutting-edge life sciences advances raise multifaceted social, legal, ethical, and security concerns, including the risk of accidental or deliberate misuse. Reconciling the expected benefits with potential risks requires effective governance of dual-use life science research. The US National Science Advisory Board for Biosecurity (NSABB), a consultative committee to the US Government defines 'dual-use research' as "the development of new technologies and the generation of information with the potential for benevolent and malevolent purposes" noting that "virtually all life sciences research has dual use potential" (1). To facilitate policy-making in this area at national as well as institutional level, the NSABB has developed a criterion for identifying dual-use research of concern (DURC): "life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security" (2, 3).

Dual-use risk management needs to be multi-layered and flexible; regulation *per se* is not sufficient to capture the wide-ranging security implications of cutting-edge life science advances. Fostering a shared understanding within the life science community of the risk that the life sciences could be misused in ways that cause harm to humans, animals, or plants is key. Life sciences stakeholders have a duty to be aware of the potential for misuse of scientific findings and of their obligation to help inform and shape critical policy decisions about biological security in the life sciences (1). On the other hand, policymakers should also seek to strike an appropriate balance between national security and unhindered scientific research when considering DURC gover-nance options.

DUAL-USE LIFE SCIENCE RESEARCH

The international norm against deliberate disease and the hostile misuse of life sciences is enshrined in the 1975 Biological and Toxin Weapons Convention (BTWC). Under Article I of the Convention:

> "Each State Party to this Convention undertakes never in any circumstances to develop, produce, stockpile or otherwise acquire or retain: (1) microbial or other biological agents, or toxins whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes" (4).

A fundamental element of the BTWC is the 'ge-neral purpose criterion' which seeks to gua-rantee a comprehensive international ban on biological and toxin weapons, on the one hand, and ensure the legitimate use of biological agents and toxins for peaceful, prophylactic, and protective purposes, on the other (5). BTWC is of relevance to the governance of dual-use life sciences research, insofar as the latter entails benignly intended (legitimate) work, which could be misused for hostile purposes. Hence, it is of utmost importance that dual-use life sciences research is considered through a broad lens, which takes into account the interdisciplinary character of such activities.

The 2006 National Research Council's report *Globalisation, Biosecurity and the Future of Life Sciences* examined trends and objectives of research in the life sciences and converging fields such as materials science and nanotechnology that may enable the development of a new generation of biological threats (6). The report noted that:

> "The growing concern regarding novel types of threat agents does not diminish the importance of naturally occurring threat agents […] or "conventionally" genetically engineered pathogenic organisms. However, it does mandate the need to adopt a **broader perspective** in assessing the threat, focusing **not** on a **narrow list** of pathogens, but on a **much wider spectrum** that includes biolo-gically active chemical agents. The potential **threat spectrum** is **thus exceptionallybroad** and **continuously evolving** – in some ways predictably, in other ways unexpec-tedly. The viruses, microbes, and toxins listed as "select agents" […] are just one aspect of this changing landscape of threats. Although some of them may be the most accessible or apparent threat agents to a potential attacker, particularly one lacking a high degree of technical expertise, this **situation is likely to change** as a result of the **increasing globalization** and **international dispersion** of the most cutting-edge aspects of life sciences research" (6). [emphases added]

A broad-based, intertwined network of mutually reinforcing actions implemented in a manner that engages a wide variety of communities are required to successfully reduce the likelihood that novel technologies may be used for malevolent purposes (6). Those in the life sciences – whether in academia, industry, or government – have an important role to play in shaping the governance ecosystem of dual-use research to ensure that biological security risks are managed in a timely and effective manner (7).

THE VALUE OF LIFE SCIENCES ENGAGEMENT

Promoting awareness of the norm against biological weapons among those in the life sciences has been recognised by BTWC States Parties as a way of ensuring the effective and comprehensive implementation of all elements of the Convention. The Eighth Review Conference of the BTWC in 2016, when considering Article IV on the national implementation of the Convention noted the value of national implementation measures to:

> "(a) implement voluntary management **standards** on **biosafety** and **biosecurity**;
> (b) encourage the consideration of development of appropriate **arrangements to promote awareness** among relevant professionals in the **private** and **public sectors** and throughout relevant scientific and administrative activities;
> (c) promote amongst those working in the biological sciences **awareness** of the **obligations** of States Parties **under the Convention**, as well as relevant **national** legislation and guidelines;
> (d) promote the development of **training and education programmes** for those granted access to biological agents and toxins relevant to the Convention and for those with the knowledge or capacity to modify such agents and toxins;
> (e) encourage the promotion of a **culture of responsibility** amongst relevant national professionals and the voluntary development, adoption and promulgation of **codes of conduct**" (8). [emphases added]

Life scientists need to be aware of the dual-use potential of their work and contribute to the identification, assessment, and mitigation of biological security risks. The Inter-Academy Partnership (IAP)'s guiding document *Doing Global Science: A Guide to Responsible Conduct in the Global Research Enterprise* contains a designated chapter on "Preventing the Misuse of Research and Technology" which elucidates the responsibility incumbent upon those in the life sciences with regard to dual-use research:

> "The difficulty of predicting the future course and applications of research does not absolve researchers of the responsibility for participating in venues to explore these issues. **Researchers need to participate in discussions about the possible consequences of their work, including harmful consequences, in planning research projects**. As the ones who design and carry out research, researchers can provide information on the nature and purpose of research that is not available in any other way" (9). [emphases as original].

In its *Responsible life sciences research for global health security: a guidance document* published in 2010, the World Health Organisation (WHO) has underscored that the consideration of issues related to possibility of accidents or misuse of the life sciences constitutes an essential element of an effective biorisk management framework for responsible life science research (10). Researchers and institutions need to develop "a better understanding of the potential risks associated with accidents and the deliberate misuse of life sciences research" and "learn about practical measures that will enable them to manage some of the risks posed by life sciences research" (10).

Shortly after the publication of this guidance document, the gain-of-function research on the genetic transmissibility of H5N1 influenza virus conducted by two groups (one in the Netherlands and the other a joint Japan/US group) prompted WHO to convene technical consultations with international experts from science, ethics, safety, and security fields to address go-vernance issues on these DURC experiments (11, 12, 13). Participants noted that:

- "DURC is an issue for **all** countries. Scientific research is conducted in virtually all countries and is critical to strengthening global response to **all health threats and hazards**, including those posed by naturally occurring and by accidentally or intentionally released biological agents.
- The management of DURC-related risks should take into account **all stages** of the research cycle, from initial conceptualization and development of a proposal, to provision of funding, to conduct of the research, analysis of results, storage and potential use of material results, including modified biological agents, and dissemination of findings.
- Some countries and institutions have developed **oversight mechanisms** to manage DURC-related risks. Many, however, have not done so, owing to competing demands on resources and capacity, limited awareness of the issue, or a perception that it is not relevant to their particular context or priorities. Nonetheless, oversight mechanisms which take into account **both the benefits** of undertaking such research as well as **the risks are important**.
- The development of guiding principles, toolkits, best practices and other forms of technical assistance would help countries formulate their own policies and procedures for managing DURC. Although establishment of a **legally** binding global agreement or re-gulation is **theoretically possible**, such an approach would be **expensive**, **slow**, likely **impractical** and would **not** necessarily **yield** the desired benefits.
- **Communication** and continuing **dialogue** across a broad range of sectors and stakeholders are **essential** to create a **culture of responsibility**, **cooperation** and **trust**. In particular, improving **mutual** understanding of the various approaches to **risk identification** and **assessment** among stakeholders will be critical to establishing that dialogue.
- **Awareness-raising**, **education** and **training** on biosafety, biosecurity and DURC are **essential** not only for researchers but also for **all sectors and stakeholders**" (11). [emphases added]

The current Intersessional Programme (2018-2020) of the BTWC seeks to advance common understanding and effective action on many of these issues, including the review of science and technology developments and identification of potential benefits and risks relevant to the Convention; biological risk assessment and management; and opportunities for life sciences engagement such as the development of codes of conduct and biosecurity education (14). Nevertheless, it is worth noting that the perceptions of risk differ among States, something evident in the fact that fewer than 5% of the 195 WHO Member States provide oversight for dual-use research with especially dangerous pathogens (15).

As a recent attempt to promote standardised guidance in this domain, in January 2019, the World Organisation for Animal Health (OIE) published *Guidelines for Responsible Conduct in Veterinary Research: Identifying, Assessing, and Managing Dual Use* which seek to raise awareness of the dual-use potential of research in ve-terinary settings and support veterinary professionals, researchers and other stakeholders to effectively identify, assess and manage dual-use implications (16). This guidance document notes that:

"researchers and institutions should integrate **dual-use risk assessment** into their existing

standard risk assessment procedures. They should exercise their **professional responsibility**, performing a continued, detailed and well-informed risk analysis for **all stages** of the propose research, from project initiation to data publication" (16) [emphases added].

The document further stresses that "the responsibility for the identification, assessment and management of dual-use implications rests to differing degrees across many stakeholders throughout the research life cycle" including researchers and their host institutions, grant and contract funders, companies, educators, scientific publishers and other communicators of research, and regulatory authorities.

TOWARD A BIOLOGICAL SECURITY CULTURE IN THE LIFE SCIENCES

The COVID-19 pandemic has shown how vulnerable the world is to natural and man-made biological threats and there is concern that COVID-19 may lead to a resurgence in interest among terrorists for biological attacks (17). Promoting a shared understanding of dual-use risks in the life sciences is a vital prerequisite for developing effective governance mechanisms and strengthening biological security now and in a post-COVID-19 world (18). Education, awareness-raising, and training are essential instruments in this process and need to be widely utilised by life sciences stakeholders for fostering biological security competence and practical skills for the identification, assessment, management, and mitigation of dual-use risks. Biological security education can serve as a means of facilitating constructive dialogue and cooperation among different disciplines and stakeholders. The *Resource Repository* set up on the BTWC website contains a range of biosafety and biosecurity resources that may assist stakeholders with the national implementation of the Convention (19). The 2019 *Guide to Training and Information Resources on the Culture of Biosafety, Biosecurity, and Responsible Conduct in the Life Sciences* offers an overview of existing educational content and training opportunities of which life sciences professionals can make use as they develop and implement biological security programmes within their institutions (20). In addition, the International Experts Group of Biosafety and Biosecurity Regulators (IEGBBR), an ad-hoc international initiative that brings together biosafety and/or biosecurity regulatory authorities from eleven countries, is currently developing a "Review of Oversight of Dual-Use in Life Sciences". This resource describing regulatory and non-regulatory oversight approaches for dual-use issues in the IEGBBR countries is intended to serve a useful reference tool for biosecurity capacity building. It takes the form of a mobile application to be launched at the end of 2021 (21).

To maximise the effectiveness of biological security education, training material needs to be paired with an appropriate delivery strategy that meets the needs of the respective training audience. This is particularly important, as "humans are not adept at making connections between disparate fields or types of knowledge, unless they are specifically helped to do so through education" (22). Active learning approaches can help individuals take control of their own learning by enhancing sense-making, self-assessment and reflection:

> "**Motivation** to learn is fostered for learners of all ages when they perceive the school or learning environment is a place where they "belong" and when the environment promotes their **sense of agency** and **purpose**" (23). [emphases added]

An example of an effective teaching and training strategy in the area of biological security is a team-based learning (TBL) (24, 25, 26). TBL is a special form of active learning, which uses a specific sequence of individual work, group work and immediate feedback to create a motivational framework in which the focus is shifted from conveying course concepts by the instructor to the application of course concepts by learners' teams (27). The TBL format is easy to use and replicate but above all, it enables learners to draw links between abstract knowledge and professional practice by learning through experience.

The value of biological security education notwithstanding, relevant additional arrangements need to be in place to ensure sustained engagement within the life sciences community with biological security. To maximise efficacy, such arrangements should be the result of implementing a flexible combination of top-down and bottom-up governance approaches, whereby top-down approaches entail international and national regulations and governmentled initiatives and bottom-up approaches entail civil society-led activities and initiatives (28).

National policies, legal acts, and government strategies and action plans are examples of top-down approaches. Mentorship and professional certification programmes administered by trade associations, biological security codes of conduct implemented by national science academies, and dual-use risk assessment methodologies and frameworks adopted at institutional level are examples of grassroots-level activities. Both sets of approaches are essential for enhancing the governance of dual-use life sciences research and strengthening the international norm of biological prohibition.

Ensuring the effective and sustainable implementation of top-down and bottom-up biosecurity governance approaches that cut across differrent professional communities requires bringing together multiple different voices, including those of women, ethnic minorities, and young people (29). To this end, greater attention needs to be given to the development of equitable and inclusive institutional, national, and international policies, procedures, and practices that encourage and support the active participation of diverse groups of people. For instance, the United Nations has urged Governments to put women's leadership and contributions at the centre of their efforts to recover from COVID-19 and to build a "better future for everyone" (30). Whilst promoting effective gender-responsive approaches to COVID-19 response and recovery is certainly commendable, women also have an important role to play in prevention and preparedness. However, they are often not equitably represented in national or global decision-making on these areas. This is problematic, for a growing body of evidence indicates that fostering diversity and inclusion (D&I) within organisations enhances their capacity for innovation and makes them more adaptive to change, which in turn can bolster their resilience in times of crisis (31). Given the multifaceted challenges posed by the ongoing advancement of the life sciences and the increasing need for biosecurity governance innovation, promoting diversity, and equity-based inclusion within the global life sciences enterprise, including enhancing the participation of women in decision-making constitutes a key element of fostering a sustainable biological security culture.

DISCLAIMER

The views expressed here are those of the authors alone. They may not represent the views of their affiliated organisation and should not be taken as an official statement or position of the affiliated organisation.

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Date of receipt of the manuscript: 08/10/2020
Date of acceptance for publication: 17/12/2020

EVENTS/ANNIVERSARIES – EVENIMENTE/ANIVERSĂRI – ÉVÉNEMENTS/ANNIVERSAIRES – СОБЫТИЯ/ЮБИЛЕИ

MIHAIL MAGDEI – ILUSTRU MEDIC, MANAGER ŞI SAVANT ÎN SĂNĂTATE PUBLICĂ!

A păstra sănătatea este o datorie morală şi religioasă, sănătatea este baza tuturor virtuţilor sociale şi ele nu mai pot fi utile atunci când nu suntem bine.

Samuel Johnson

Dl Mihail MAGDEI, după absolvirea Institutului de Stat de Medicină din Chişinău, şi-a început activitatea profesională în anul 1969. Graţie profesionalismului, dedicaţiei şi muncii asidue a avansat rapid pe scara profesională şi ierarhică în cadrul Serviciului Sanitaro-Epidemiolopgic de Stat din republică – de la funcţia de medic epidemiolog, la cele de şef de secţie şi medic – şef al sanepidului raional Criuleni (1971-1974), medic-şef adjunct la Staţia Sanitaro-Epidemiologică Republicană (1974-1975), şef Direcţie Sanitaro-Epidemiologică la Ministerul Ocrotirii Sănătăţii (1974-1983), medic – şef la Staţia Sanitaro-Epidemiologică Republicană (1983-1994), viceministru al Sănătăţii şi medic – şef sanitar de stat al Republicii Moldova (1994-1997 şi 2009 – 2012), ministru al Sănătăţii (1997-1998), director general al Centrului Naţional Ştiinţifico – Practic de Medicină Preventivă (1998-2003).

Dl Mihail Magdei, permanent şi cu brio a făcut faţă tuturor provocărilor timpului, remarcându-se drept unul dintre cei mai notorii organizatori, manageri şi savanţi din cadrul Serviciul de Supraveghere de Stat a Sănătăţii Publice (SSSSP). Din primii ani de la proclamarea Independenţei Republicii Moldava s-a angajat plenar la menţinerea şi dezvoltarea potenţialului Serviciului pentru care a iniţiat, propus şi implementat mai multe reforme instituţionale. Permanent a străduit întru ameliorarea sănătăţii populaţiei, inclusiv prin alinierea activităţii instituţiilor din cadrul SSSSP la standardele europene.

De rând cu activitatea practică, dl Mihail Magdei a explorat şi în domeniul ştiinţei medicale, rezultatele studiilor fiind prezentate în peste 125 lucrări ştiinţifico-practice, inclusiv 2 monografii, 3 brevete de invenţii. Multiple articole şi interviuri au fost publicate în presa scrisă sau difuzate la radio şi TV.

Activitatea rodnică şi rezultatele de performanţă ale dlui Mihail Magdei în domeniul sistemului sănătăţii au fost înalt apreciate de conducerea Ministerului Sănătăţii (MS) şi a Republicii Moldova, acesta fiind menţionat de nenumărate ori cu diplome, conferit titlul onorific Om Emerit (1995) şi decorat cu Ordinul de Onoare (2011).

Mulţi ani prosperi, Domnule Mihail MAGDEI!

Cu profund şi deosebit respect, consiliul de redacţie al Revistei *One Health & Risk Management*