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MESENCHYMAL STEM CELLS VERSUS COVID-19. CAN THEY WIN THE BATTLE?

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ABSTRACT

Mesenchymal stem cells (MSCs) are multipotent stem cells with numerous features potentially useful in various pathologies. It has been shown that MSCs have regenerative potential due to modulation of immune system response, inflammation diminishing, trans differentiation into various types of cells, proangiogenetic and anti fibrotic influence. Besides all of these traits, MSCs posses anti viral capacity and have been further employed in clinical trails since last year. Here, we revised immunomodulatory, biological and antiviral traits of MSCs, but also pathogenesis of Covid-19 and it's impact on immune system. Conspicuously, there is a grow-ing number of studies examining effect of MSCs in patients suffer-ing from Covid-19 pneumonia and ARDS. Since MSCs are in theo-ry capable of healing lung injury and inflammation, here we dis-cuss hypothesis, pros and cons of MSCs treatment in Covid-19 pa-tients. Finally, we debate if MSCs based therapy can be promising tool for Covid-19 lung pathologies.

Keywords: *Mesenchymal stem cells, inflammation, covid-19, pneumonia, ARDS.*

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ABBREVIATIONS

ACE2 - Angiotensin-converting enzyme 2
AngII - Angiotensin 2 pathway
ARDS - Acute respiratory distress syndrome
AT-MSCs - Adipose tissue derived mesenchymal stem cells
BM-MSC - Bone marrow derived mesenchymal stem cells
CCL2 - C-C Motif Chemokine Ligand 2)
COPD - Chronic Obstructive Pulmonary Disease
CRP - C-reactive protein
CVDs - Cardiovascular disease
DCs- Dendritic cells
FGF7- Fibroblast Growth Factor 7
G-CSF - Alpha granulocyte-colony stimulating factor
GM-CSF- Granulocyte-macrophage colony-stimulating factor
GVHD - Graft- versus host disease
HCoV-19- Human coronavirus 2019
hESC - Human embryonic stem cell
HGF - Hepatic growth factor
hiPSC - Human induced pluripotent stem cells
HLA-G- Human leukocyte antigen-G
HO- Heterotopic ossification
HO-1- Hemeoxygenase-1
hWJCs - Wharton Jelly's MSCs
IDO - Indolamine 2,3-dioxygenase IL-10,
IFI6 - Interferon Alpha Inducible Protein 6
IFITM - Interferon-inducible transmembrane
IFNAR2 - Interferon Alpha And Beta Receptor Subunit 2
IL-1Ra - Interleukin 1 receptor antagonist
IL-6 Amp - Interleukin-6 amplifier
IL-6 - Interleukin-6
ISG - Interferon stimulated genes
KGF - Keratinocyte growth factor
LIF - Leukocyte inhibitory factor
MCP-1- Monocyte chemoattractant protein-1
MERS - Middle East respiratory syndrome
MIP1A - Macrophage inflammatory protein 1
MODS - Multiple organ dysfunction syndrome
MSC-EV- Mesenchymal stem cell derived extracellular vesicles
MSCs -Mesenchymal stem cells
MT1G -Metallothionein 1G
NETs -Neutrophil extracellular traps
NO - Nitric oxide
p21/CDKN1A - p21 Cyclin Dependent Kinase Inhibitor 1A
PD - programmed cell death
PGE2 - Prostaglandin E2
PMAIP1 - Phorbol-12-Myristate-13-Acetate-Induced Protein 1
SARS - Severe acute respiratory syndrome
SARS-CoV-2- SARS coronavirus 2
SAT- Spermidine/spermine N(1)-acetyltransferase
SERPINE1 - Serpin family E member 1
sIL-6Ra - Interleukin-6a
SARS-CoV-2S - spike glycoprotein
TGF-b - Transforming growth factor-b ,
TLRs - Toll like receptors
TMPRSS2 - Transmembrane protease serine 2
TNF-α - Tumor necrosis factor-α
Tregs - Regullatory T cells
TSG-6 - Tumor necrosis factor a-stimulated gene 6
UCB-MSCs - Umbilical chord blood derived mesenchymal stem cells
VEGF - Vascular endothelial growth factor
WHO -World Health Organization

INTRODUCTION

Mesenchymal stem cells (MSCs) are adult multipotent stem cells with ability to self renew and can be isolated from various tissues (1). MSCs are defined by three norms- adherence to culture flasks; expression of cluster of differentiation CD105, CD73 and CD90 but also absence of CD45, CD34, CD11b, CD14, CD79a, CD31 and MHC class II molecule. Additionally, MSCs can differentiate into different types of tissues (2) therefore representing an excellent tool for regenerative medicine. MSCs can migrate to the sites of injury and modulate immune response (3). This trait, among others, makes MSCs an excellent choice for immune-mediated diseases. Immediately after engraftment, MSCs interact with both, innate and adaptive immune system in two ways; by paracrine manner and by PD receptor interaction. Paracrine manner includes release of numerous soluble factors- transforming growth factor-b (TGF-b), hepatic growth factor (HGF), nitric oxide (NO), indolamine 2,3-dioxygenase (IDO), IL-10, IL-6, leukocyte inhibitory factor (LIF), IL-1 receptor antagonist (IL-1Ra), galectins, tumor necrosis factor a-stimulated gene 6 (TSG-6), human leukocyte antigen-G (HLA-G), hemeoxygenase-1 (HO-1), and prostaglandin E2 (PGE2) hence emphasizing it's immunosupressive potential. MSCs influence immune response by interaction with crucial inflammatory cells, specifically, MSCs abolish production and spur of pro-inflammatory M1 macrophages and provoke their alteration in M2 phenotype (4). Also, MSCs can negatively impact dendritic cells DCs in terms of their growth and secretion of various cytokines, which eventually leads to diminished inflammation due to T cells inhibition (5-7). MSCs can diminish generation of Th1 and Th17 cytokines, but also enhance Th2 cytokines generation (19–21). Proliferation of Th1 and Th17 cells can be suppressed by MSCs, but also,

MSCs can add to the number of Tregs, and in that manner can have immunosupressive and anti-inflammatory effect (8). In spite of that, MSCs can adopt pro-inflammatory phenotype as well, which is determined by the environmental conditions (9). Actually, low levels of pro inflammatory cytokines promote pro-inflammatory phenotype of MSCs and vice versa (9). This balance of MSCs' phenotype is determined by Toll like receptors (TLRs) (10) that also represent main receptor for virus recognition (11-13). Important element of MSCs mediated immunomodulation are exosomes (14,15) and various secretomes (16,17). For instance, Let-7, miR-34a, miR-146a and miR200b/c are known to be involved in proinflammatory metabolic pathways (18,19). Let-7 has been involved in the suppression of posttranscriptional regulation of IL-6 secretion and downregulates TLR4 signal (20-22). Also, miR-34a and miR-146 turns out to have a critical role in regulation of NF-kB cascade, possibly aiming upstream elements at the time of T cell stimulation (23,24). Eventually, miR-200b/c presumably diminishes complement tentative cytolysis, induced by C5b-9 coupling, since suppression of miR-200c increased MSC demise (25). These outcomes propose the anti inflammatory and cytoprotective features of miRNAs from MSC-EV, confirming that MSCs secretomes promote tissue regeneration by neoangiogenesis, apoptosis inhibition and fibrosis inhibition (26-32), which can be beneficial in numerous pathologies especially those with inflammatory component in pathogenesis.

MSCs sources

Bone marrow, adipose tissue, umbilical cord and dental pulp are suggested as convenient sources of MSCs, yet it is not completely elucidated which source of MSCs is optimal (38, 39). BM-MSCs are characterized by easy acquisition, rapid expansion *in vitro*, minimum immunologic rejection, protracted standing post-transplatationally in the host, preservation of capacity to differentiate subsequently to repetitive passages as well as simplicity of transplantation (40,41). Anyway, the acquisition of BM-MSCs is complicated process and also, size and longevity of BM-MSCs diminishes by ageing (42,43). So, to bypass such obstacles, alternative sources for MSCs isolation are implied (12,13). Umbilical cord blood and adipose tissue have been proposed as an alternate source for isolation and therapeutic employment of MSCs regarding its larger proliferative potential, high cell yields and ease of harvesting (44,45). BM-MSCs, UCB-MSCs and AT-MSCs all have similar morphological as well as operational traits (11). In the context of current pandemic Covid-19, and the entire inflammatory response caused by cytokine storm, as it will be further elaborated in this review, adequate MSCs source should be discussed so that optimal MSCs could be employed in therapy. Since the virus enters the cells through target ACE2 receptor, it is crucial to decide whether MSCs from any of these sources express ACE2 receptor, and therefore is susceptible to Covid-19 infection. Recent study showed that ACE2 is highly expressed in adult bone marrow, adipose tissue or umbilical cord-derived MSC. Distinguishing from placental derived MSCs together with human embryonic stem cell (hESC) or human induced pluripotent stem cells (hiPSC) that express low levels of ACE2 receptor (46). These outcomes should be further taken into consideration when determining on the optimal source of MSC therapy.

Cytokine storm in Covid-19

In December 2019, non typical infectious respiratory syndrome of idiopathic cause was recognised in Wuhan, China. Having already met with manifestations of (SARS) in 2003, Chinese scientific and medical department could identify a new coronavirus, SARS coronavirus 2 (SARS-CoV-2) as the pathogen (47). SARS-CoV-2 was easily and quickly spread all across the globe, leading to high morbidity and mortality. In order to prevent further spread of the virus, lock-down and other quarantine measures were enforced. Ease of spreading, high morbidity and mortality rate, changed the portrait of this pathogen, so on March 11, 2020, the World Health Organization (WHO) declared COVID-19 a global pandemic. The COVID-19 is clinically heterogeneous, patients may be asymptomatic or have light, moderate, severe or critical clinical traits. Symptoms of COVID-19 may vary, but most common include fever, headache, malaise, cough, bone pain, myalgias, anosmia, impaired taste and respiratory distress. Similar to SARS in 2003, this infectious disease leads to a high probability of ICU admission and mortality (48, 49). The pathogenesis of SARS-CoV-2 has been explained by the interaction and binding of the virus spike glycoprotein (SARS-CoV-2S) to the angiotensin-converting enzyme-related carboxypeptidase (ACE2) on the target cell surface. After binding, spike protein is activated by the cellular transmembrane protease serine 2 (TMPRSS2) which enables the virus to enter the host cell (50), leading to complex over activated inflammation and immune response, known as cytokine storm. The term "cytokine storm" was first established in 1993. to depict a graft versus host disease, and defines as the rapid and sudden efflux of a large number of cytokines that happens as a result of the immune system′s overactivation by some stimuli (infection, drugs, etc.), begin locally and disseminate systemically, triggering collateral detriment in tissues (51,52). It is elucidated that cytokine storm is linked with the exacerbation of various infectious diseases, such as severe acute respiratory syndrome (SARS), Middle East respiratory syndrome (MERS), and with the severity of COVID-19 (53). However, it was reported that cytokine storm is one of the main causes of a severe form of the disease in COVID-19 patients and dying, and is associated with thrombosis, massive mononuclear cell infiltration in multiple organs, and high levels of circulating cytokines (54). Notwithstanding that the specific dysregulated molecular causes are still not understood, it is assumed that cytokine storm is caused by disbalance in the regulation of the immune system (i.e., increment in immune cell activation via TLR or another mechanism, decreasing in anti-inflammatory response, etc.) (51). The immune response is being provoked by SARS-CoV-2 entering respiratory epithelial cells and is accompanied by inflammatory cytokine production and weak interferon (IFN) response. Cytokine storm occurs as a result of activation of pro-inflammatory Th1 cells and intermediate CD14+ CD16+ monocytes, macrophages, and neutrophils infiltration into the

lung tissue (54). Specifically, pathogenic Th1 cells that produce pro-inflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-6 (IL-6) can be activated by SARS-CoV-2 very fast. After that, GM-CSF further activates CD14+ CD16+ inflammatory monocytes to secrete immense amounts of IL-6 and tumor necrosis factor-α (TNF-α) (55). Neutrophil extracellular traps (NETs), weak IFN-γ induction, and membrane-bound immune receptors (e.g., Fc and Toll-like receptors), might be some of the causes for massive cytokine release (53,54). One of the possible mechanisms of the cytokine storm is induced by the angiotensin 2 (AngII) pathway, where SARS-CoV-2 triggers NF-κB. ACE2 on the cell surface is occupied by SARS-CoV-2, which results in decreasing in ACE2 expression and an increase in AngII. Also, AngII-angiotensin receptor type 1 (AngII-AT1R) axis can induce TNF-α and the soluble form of IL-6Ra (sIL-6Ra). IL-6 binds to sIL-6R through gp130 to form the IL-6-sIL-6R complex, which can trigger STAT3 in non-immune cells. Both NF-κB and STAT3 are able for activation of the IL-6 amplifier (IL-6 Amp) to induce various pro-inflammatory cytokines and chemokines, such as IL-8, and IL-6, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1) (56, 57). Some studies have been demonstrated that levels of IL-1β, IL-2, IL-6, IL-7, IL-8, IL-12, inducible protein 10 (IP-10), MCP-1, TNF-α, macrophage inflammatory protein 1 alpha (MIP1A), granulocyte-colony stimulating factor (G-CSF), and IFN-γ were increased, though levels of the Th2 cytokine IL-4 were low in patients with severe COVID-19 (58, 59,60). Other studies showed that the level of CD4＋cells and CD8＋ cells was reduced constantly, but neutrophil counts were elevated in patients with a severe form of COVID-19 comparing with mild patients (60). Because of the rapid evolution of the cytokine storm, critically ill COVID-19 patients develop acute respiratory distress syndrome (ARDS) (61). ARDS is described as a multi-factorial syndrome of severe lung injury whose main characteristics are: hypoxemia, pulmonary oedema, diffuse alveolar damage and multiple organ failure. Lungs are not able to provide sufficient oxygen saturation to the alveolar spaces and respiratory support may be required (62). Yet, there are paradoxical outcomes in terms of SARS-CoV-2 positive Chronic Obstructive Pulmonary Disease (COPD) patients. Actually, most of COPD patients did not develop critical symptoms, which is contradictory, due to the fact that pulmonary co morbidity elevates chances of ARDS development and fatal outcome (63, 64). It is assumed that key role in this “protection” plays IL-6. Covid-19-related pneumonia is caused by inflammasomes activation and secretion of IL-1, TNF and IL-6, making IL-6 crucial in ARDS development. IL-6 coordinates the cytokine storm resulting in an immune hyper-reaction and in the inflammatory injury of the pulmonary tissue (65). This is in line with a *meta*-analysis that emphasized high levels IL-6 in the severe forms of Covid-19 (66). Moderately-elevated concentrations of IL-6 found in moderate COPD patients has protective roles against the decay of Covid-19, explaining therefore their better prognosis. Eventually, the drug Tocilizumab, a monoclonal antibody that blocks the IL-6 receptor, is currently used to treat Covid-19 in hospitals (67). These outcomes, lead to conclusion that Tocilizumab must be utilised only in patients with an current cytokine storm, or else, it’s usage may be damaging.

MSCs’ antiviral traits against Covid-19

Luckily, MSCs are insusceptible to virus infections due to IFN-stimulated genes (ISG), that can impact every of step of viral cycle (68-70). Specifically, PMAIP1, ISG15, IFI6, IFITM, SAT1, p21/CDKN1A, SERPINE1 and CCL2 are ISGs that being expressed in MSCs are shown to halt numerous viral pathogens, but most importantly SARS (69). Team of researches made a list of ISGs constitutively expressed by human MSC and afterwards set up by IFITM1, IFI6, CCL2, ISG15, SAT1 and PMAIP1. In attendance of IFN-γ there was an induction of non-constitutive ISGs, as well as MT1G, CD74, SERPING1, IFNAR2 and MT1X (68). MSCs have this great ability to switch to non-constitutive ISGs up regulation in order to better fight viruses, which can be advantageous in regards to respiratory infections (68). One of the most crucial ISGs is IDO, which not just that have immunomodulatory properties, but also inhibits viral protein bio synthesis (71). These anti viral features, besides familiar MSCs’ immunomodulatory, antiinflammatory, neoangiogenic, regenerative and multipotent potential, definitely make MSCs potent and competent player in a harsh battle against Covid-19.

MSCs as a tool for Therapeutic Applications in Covid-19

Since SARS-CoV-2 and COVID-19 first and foremost affect lungs, leading to ARDS, pneumonia and MODS consecutively, plus the fact that MSCs entrap in lungs, traits of MSCs residing in lungs should be thoroughly observed as therapeutic tool for SARS-CoV-2. It is shown that lung residing MSCs promote regeneration and tissue repair (72). Lung residing MSCs are positioned perivasculary and express CD73 which in turn promotes expression of anti-inflammatory genes in MF and vice versa (72). Again, exhibition of this marker is flexible and differ depending on the cell surrounding conditions, and testify how MSCs are sensitive to inflammatory response (73). MSCs respond by production of soluble factors, specifically when in lungs- MSCs secrete FGF7 (or KGF) leading to normalization of alveolar clearance (74). Various proteomic and transcriptomic analyses displayed that lung MSCs affect signal pathways in regards to up regulation of Wnt/β-catenin and down-regulation of NF-κB signaling, leading to diminished TNF-α production and eventually to fibrosis reduction (75, 76, 77). MSCs displayed remedial effect in model of ARDS by stimulating surfactant secretion, differentiating into pulmonary endothelial cells and alveolar epithelial cells (78). Hence, most important are exactly these effect in regards to SARS-CoV-2-cytokine storm and ARDS (79, 80), which has already been elaborated in preclinical and clinical studies (81, 82).

Clinical applications of MSCs in COVID-19

Most recent study revised outcomes of MSCs applied in patients with ARDS and Covid-19 related conditions (83).

When it comes to ARDS, highlights should be noted; patients with ARDS received BM-MSCs in dose of 10 million cells/kg. It is found no MSCs related adverse effects, except for, three patients who died in next weeks after treatment. Yet, for one who died from multiple spleen, brain and kidney embolic infarctions, it is confirmed by MRI that these changes were present prior to MSC administration (84) suggesting MSCs administration as safe. This study was the first phase of research, that was extended by Matthay et al. (85), and included much larger group of moderate to severe ARDS patients who received intravenously same dosage of human bone-marrow-derived MSCs. This study suggested no infusion-related haemodynamic or respiratory complications, even though higher numerically mortality and severity scores were observed in patients who received MSCs comparing to placebo group, but this difference was not statistically significant. Most important is the finding that showed direct impact of MSCs viability on angiopoetin 2 plasma concentrations, leading to drastic decrease of angiopoetin 2 concentrations, 6h after MSCs infusion which can be beneficial in ARDS. This study is planned to be continued, so more consistent and clear data could enlighten previous conclusions. In spite of insufficient and limited data in regards to clinical outcomes from patients that received MSCs therapy to fight Covid-19 respiratory disease, certain clinical trials emerged and propose MSCs as safe, beneficial and with no detrimental adverse effects (86, 87). Patients in China, on March in 2020, with severe COVID-19 pneumonia, were treated with MSCs, intravenously. In this study for the first time it is showed that MSCs are ACE2- and TMPRSS2- and that MSCs secrete anti-inflammatory factors to prevent the cytokine stormhence displaying that MSCs have natural immunity to the HCoV-19. This MSCs therapy improved vital condition of patients, reduced level of inflammatory cytokines and chemokines so less mononuclear/ macrophages were attracted to injured lung tissue. At the same time bigger number of regulatory DCs were directed to the inflammatory tissue niche. In addition, they noted enhanced levels of IL-10 and VEGF, decreased C-reactive protein and TNF-α levels (80). Among these data most important was the observation of lack of overactivated cytokine-secreting immune cells CXCR3+ CD4+ T cells, CXCR3+ CD8+ T cells and CXCR3+ NK cells (80), which in summary can explain lung regeneration and improvement in critical patients. Later on, in June, a detailed review by Rajarshi stated MSCs therapy in COVID-19 patients as justified and advantageous (88). Other study examined therapeutic potential of Wharton Jelly's MSCs (hWJCs) delivered intravenously in COVID-19 positive critically ill patient with pneumonia and diabetes. After his vital parameters stabilized, he received hWJCs intravenously. After hWJC adoptive transfer, there were no adverse effects whereas level of serum CRP and inflammatory factors (IL-6 and TNF-α) were drastically decreased, and vital parameters of the patient improved. Plus the level of CD3+, CD4+ and CD8+ T cell were significantly increased after intravenous injection of hWJCs (89). This is the first study of this kind, and though encouraging and promising, should be elaborated and further examined with much bigger group of patients. These preliminary clinical records lead to conclusion that MSCs alone or combined with other therapeutics could enhance the chances of survival and improve overall health condition in Covid-19 patients (80,90). One study examined exosomes (ExoFloTM) derived from allogeneic BM-MSCs as treatment for severe COVID-19 (91). 24 SARS-CoV-2 PCR positive patients received 15 mL dose of ExoFlo intravenously and were observed in regards to safety and effectiveness.

There were no any side effects of ExoFlo treatment 72 hours after treatment. 83% of the patients survived, 71% of patients cured; 13% stayed in critical though stable condition; 16% passed away of causes unconnected to the therapy. On the whole, after treatment, patients' clinical condition and oxygenation improved. Additionally, there were enhancements in absolute neutrophil number and lymphopenia. Also, there was significant diminishing of C-reactive protein (CRP), Ferritin and D-dimer, confirming therefore beneficial effect of ExoFlo on inflammatory response. Having in mind such data, ExoFlo could be a prommising tool of Covid-19 related pathology, due to it's safety, ability to halt inflammation, stabilize immune response and oxygenation. In spite of that, further research with bigger group of patients with similar results could guarantee ExoFlo as secure therapeutic approach.

Ongoing Clinical trials MSC-Based Therapies for COVID-19

In order to summarize ongoing MSC-based therapies in patients suffering from Covid-19 worldwide ClinicalTrials.gov database was analysed. There are 68 studies that include MSCs treatment in various Covid-19 related conditions-Pneumonia and ARDS. Most of these studies are randomised and open-label studies and in early phase (phase I, I/II, II). Specifically, 29 of the studies are currently recruiting, yet 26 are not recruiting yet. There are 8 completed studies, while there are 3 enrolling and 1 study is withdrawn. Most of the studies utilised BM-MSC, UC-MSC, WJ MSC, AT-MSCs. However, there are fewer studies that utilised allogeneic dental pulp MSCs, placenta-derived MSCs, stromal MSCs, MSC derived exosomes and secretomes. Most of the studies follow basic protocols of disease severity assessment such as inflammatory cytokine profile (TNF-α, IL-6 and IL-10), vital parameters and pulmonary function assessment. Studies groups include both, males and females varying in age. All of these data are summarized in Table 1.

Table 1. MSC-based ongoing clinical trials for Covid-19 infection diseases

Study Name:	Mesenchymal Stem Cell Infusion for COVID-19 Infection (NCT04444271)
Status:	Recruiting (Phase II)
Treatment:	MSC

Study Name:	Mesenchymal Stem Cell for Acute Respiratory Distress Syndrome Due for COVID-19 (NCT04416139)
Status:	Recruiting (Phase II)
Treatment:	MSC

Study Name:	Mesenchymal Stem Cells Therapy in Patients With COVID-19 Pneumonia (NCT04713878)
Status:	Completed
Treatment:	MSC

Study Name:	Safety and Efficacy of Mesenchymal Stem Cells in the Management of Severe COVID-19 Pneumonia (NCT04429763)
Status:	Not yet recruiting (Phase II)
Treatment:	UC-MSC

Study Name:	Cord Blood-Derived Mesenchymal Stem Cells for the Treatment of COVID-19 Related Acute Respiratory Distress Syndrome (NCT04565665)
Status:	Recruiting (Phase I)
Treatment:	MSC

Study Name:	NestaCell® Mesenchymal Stem Cell to Treat Patients With Severe COVID-19 (NCT04315987)
Status:	Not yet recruiting (Phase II)
Treatment:	MSCs (NestaCell®)

Study Name:	Mesenchymal Stem Cells in Patients Diagnosed With COVID-19 (NCT04611256)
Status:	Recruiting(Phase I)
Treatment:	MSC

Study Name:	Use of Mesenchymal Stem Cells in Acute Respiratory Distress Syndrome Caused by COVID-19 (NCT04456361)
Status:	Early Phase I
Treatment:	WJ-MSC

Study Name:	Efficacy of Infusions of MSC From Wharton Jelly in the SARS-Cov-2 (COVID-19) Related Acute Respiratory Distress Syndrome (NCT04625738)
Status:	Not yet recruiting (Phase II)
Treatment:	WJ-MSC

Study Name:	Mesenchymal Stem Cell Treatment for Pneumonia Patients Infected With COVID-19 (NCT04252118)
Status:	Recruiting(Phase I)
Treatment:	MSCs

Study Name:	Clinical Trial of Allogeneic Mesenchymal Cells From Umbilical Cord Tissue in Patients With COVID-19 (NCT04366271)
Status:	Recruiting (Phase II)
Treatment:	WJ-MSC

Study Name:	Treatment of COVID-19 Patients Using Wharton's Jelly-Mesenchymal Stem Cells (NCT04313322)
Status:	Recruiting(Phase I)
Treatment:	Use of Stem Cells for COVID-19 Treatment WJ-MSCs

Study Name:	Clinical Trial to Assess the Safety and Efficacy of Intravenous Administration of Allogeneic Adult Mesenchymal Stem Cells of Expanded Adipose Tissue in Patients With Severe Pneumonia Due to COVID-19 (NCT04366323)
Status:	Active, not recruiting (Phase II)
Treatment:	AT-MSC

Study Name:	Treatment of Severe COVID-19 Patients Using Secretome of Hypoxia-Mesenchymal Stem Cells in Indonesia (NCT04753476)
Status:	Recruiting (Phase II)
Treatment:	Injection of Secretome-MSCs

Study Name:	Safety and Efficacy Study of Allogeneic Human Dental Pulp Mesenchymal Stem Cells to Treat Severe COVID-19 Patients (NCT04336254)
Status:	Recruiting (Phase I, II)
Treatment:	Allogeneic human dental pulp stem cells (BSH BTC & Utooth BTC)|

Study Name:	Bone Marrow-Derived Mesenchymal Stem Cell Treatment for Severe Patients With Coronavirus Disease 2019 (COVID-19) (NCT04346368)
Status:	Not yet recruiting (Phase I, II)
Treatment:	BM-MSCs

Study Name:	Treatment With Human Umbilical Cord-derived Mesenchymal Stem Cells for Severe Corona Virus Disease 2019 (COVID-19) (NCT04288102)
Status:	Completed (Phase II)
Treatment:	UC-MSCs

Study Name:	Study to Evaluate the Efficacy and Safety of AstroStem-V in Treatment of COVID-19 Pneumonia (NCT04527224)
Status:	Not yet recruiting (Phase I, II)
Treatment:	Adipose tissue-derived mesenchymal stem cells (AstroStem-V)

Study Name:	Regenerative Medicine for COVID-19 and Flu-Elicited ARDS Using Longeveron Mesenchymal Stem Cells (LMSCs) (NCT04629105)
Status:	Recruiting (Phase I)
Treatment:	Longeveron Mesenchymal Stem Cells (LMSCs)

Study Name:	Study of Human Umbilical Cord Mesenchymal Stem Cells in the Treatment of Severe COVID-19 (NCT04273646)
Status:	Not yet recruiting
Treatment:	UC-MSCs

Study Name:	Study of Intravenous Administration of Allogeneic Adipose-Derived Mesenchymal Stem Cells for COVID-19-Induced Acute Respiratory Distress (NCT04728698)
Status:	Not yet recruiting (Phase II)
Treatment:	COVI-MSC

Study Name:	A Randomized, Double-Blind, Placebo-Controlled Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Allogeneic Mesenchymal Stem Cell Therapy (HB-adMSCs) to Provide Protection Against COVID-19 (NCT04348435)
Status:	Enrolling by invitation (Phase II)
Treatment:	HB-adMSCs

Study Name:	Treatment of Covid-19 Associated Pneumonia With Allogenic Pooled Olfactory Mucosa-derived Mesenchymal Stem Cells (NCT04382547)
Status:	Enrolling by invitation (Phase I,II)
Treatment:	Allogenic pooled olfactory mucosa-derived MSC

Study Name:	Clinical Research of Human Mesenchymal Stem Cells in the Treatment of COVID-19 Pneumonia (NCT04339660)
Status:	Recruiting(Phase I,II)
Treatment:	UC-MSCs

Study Name:	Expanded Access Protocol on Bone Marrow Mesenchymal Stem Cell Derived Extracellular Vesicle Infusion Treatment for Patients With COVID-19 Associated ARDS (NCT04657458)
Status:	Phase I
Treatment:	BM-MSC Derived Extracellular Vesicles Infusion Treatment

Study Name:	Mesenchymal Stem Cell Therapy for SARS-CoV-2-related Acute Respiratory Distress Syndrome (NCT04366063)
Status:	Recruiting(Phase II,III)
Treatment:	Cell therapy protocol

Study Name:	Autologous Adipose-derived Stem Cells (AdMSCs) for COVID-19 (NCT04428801)
Status:	Not yet recruiting(Phase II)
Treatment:	Autologous adipose-derived stem cells

Study Name:	Administration of Allogenic UC-MSCs as Adjuvant Therapy for Critically-Ill COVID-19 Patients (NCT04457609)
Status:	Recruiting (Phase I)
Treatment:	Oseltamivir| UC-MSC

Study Name:	A Clinical Trial to Determine the Safety and Efficacy of Hope Biosciences Autologous Mesenchymal Stem Cell Therapy (HB-adMSCs) to Provide Protection Against COVID-19 (NCT04349631)
Status:	Active, not recruiting (Phase II)
Treatment:	HB-adMSCs

Study Name:	Adipose Mesenchymal Cells for Abatement of SARS-CoV-2 Respiratory Compromise in COVID-19 Disease (NCT04352803)
Status:	Not yet recruiting(Phase I)
Treatment:	Autologous Adipose MSC's

Study Name:	Mesenchymal Stem Cells for the Treatment of COVID-19 (NCT04573270)
Status:	Completed(Phase I)
Treatment:	UC-MSC (PrimePro)

Study Name:	Novel Coronavirus Induced Severe Pneumonia Treated by Dental Pulp Mesenchymal Stem Cells (NCT04302519)
Status:	Not yet recruiting(Phase I)
Treatment:	Dental pulp MSC

Study Name:	Umbilical Cord Tissue (UC) Derived Mesenchymal Stem Cells (MSCs) Versus Placebo to Treat Acute Pulmonary Inflammation Due to COVID-19 (NCT04490486)
Status:	Not yet recruiting(Phase I)
Treatment:	UCMSCs

Study Name:	Use of UC-MSCs for COVID-19 Patients (NCT04355728)
Status:	Completed (Phase I,II)
Treatment:	Umbilical Cord Mesenchymal Stem Cells + Heparin along with best supportive care.

Study Name:	An Exploratory Study of ADR-001 in Patients With Severe Pneumonia Caused by SARS-CoV-2 Infection (NCT04522986)
Status:	Not yet recruiting (Phase I)
Treatment:	MSC

Study Name:	Treatment of Coronavirus COVID-19 Pneumonia (Pathogen SARS-CoV-2) With Cryopreserved Allogeneic P_MMSCs and UC-MMSCs (NCT04461925)
Status:	Recruiting (Phase I,II)
Treatment:	Placenta-Derived MMSCs; Cryopreserved Placenta-Derived Multipotent Mesenchymal Stromal Cells

Study Name:	Safety and Effectiveness of Mesenchymal Stem Cells in the Treatment of Pneumonia of Coronavirus Disease 2019 (NCT04371601)
Status:	Active, not recruiting (Phase I)
Treatment:	Oseltamivir mesenchymal stem cells

Study Name:	BAttLe Against COVID-19 Using MesenchYmal Stromal Cells (NCT04348461)
Status:	Not yet recruiting (Phase II)
Treatment:	Allogeneic and expanded adipose tissue-derived Mesenchymal stromal cells

Study Name:	Efficacy and Safety Study of Allogeneic HB-adMSCs for the Treatment of COVID-19 (NCT04362189)
Status:	Active, not recruiting (Phase II)
Treatment:	HB-adMSC

Study Name:	Therapeutic Study to Evaluate the Safety and Efficacy of DW-MSC in COVID-19 Patients (NCT04535856)
Status:	Completed(Phase I)
Treatment:	Allogeneic mesenchymal stem cell

Study Name:	Therapy for Pneumonia Patients iInfected by 2019 Novel Coronavirus (NCT04293692)
Status:	Withdrawn
Treatment:	UC-MSCs

Study Name:	Safety and Efficacy of Intravenous Wharton's Jelly Derived Mesenchymal Stem Cells in Acute Respiratory Distress Syndrome Due to COVID 19 (NCT04390152)
Status:	Recruiting (Phase I,II)
Treatment:	WJ-MSC

Study Name:	Study of the Safety of Therapeutic Tx With Immunomodulatory MSC in Adults With COVID-19 Infection Requiring Mechanical Ventilation (NCT04397796)
Status:	Recruiting(Phase I)
Treatment:	BM-Allo.MSC

Study Name:	A Phase II Study in Patients With Moderate to Severe ARDS Due to COVID-19 (NCT04780685)
Status:	Recruiting (Phase II)
Treatment:	hMSC

Study Name:	Umbilical Cord Lining Stem Cells (ULSC) in Patients With COVID-19 ARDS (NCT04494386)
Status:	Recruiting (Phase I,II)
Treatment:	Umbilical Cord Lining Stem Cells (ULSC)

Study Name:	Mesenchymal Stem Cells (MSCs) in Inflammation-Resolution Programs of Coronavirus Disease 2019 (COVID-19) Induced Acute Respiratory Distress Syndrome (ARDS) (NCT04377334)
Status:	Not yet recruiting (Phase II)
Treatment:	MSC

Study Name:	Use of hUC-MSC Product (BX-U001) for the Treatment of COVID-19 With ARDS (NCT04452097)
Status:	Not yet recruiting(Phase I,II)
Treatment:	Human umbilical cord mesenchymal stem cells

Study Name:	Mesenchymal Stromal Cells for the Treatment of SARS-CoV-2 Induced Acute Respiratory Failure (COVID-19 Disease) (NCT04345601)
Status:	Recruiting (Phase I,II)
Treatment:	Mesenchymal Stromal Cells

Study Name:	Efficacy and Safety Evaluation of Mesenchymal Stem Cells for the Treatment of Patients With Respiratory Distress Due to COVID-19 (NCT04390139)
Status:	Recruiting (Phase I,II)
Treatment:	Expanded MSC from Wharton Jelly (XCEL-UMC-BETA)

Study Name:	Investigational Treatments for COVID-19 in Tertiary Care Hospital of Pakistan (NCT04492501)
Status:	Completed
Treatment:	Convalescent Plasma|Drug: Tocilizumab|Drug: Remdesivir|MSC

Study Name:	Clinical Use of Stem Cells for the Treatment of Covid-19 (NCT04392778)
Status:	Recruiting(Phase I,II)
Treatment:	MSC Treatment

Study Name:	Safety and Feasibility of Allogenic MSC in the Treatment of COVID-19 (NCT04467047)
Status:	Not yet recruiting (Phase I)
Treatment:	Mesenchymal Stromal Cells infusion

Study Name:	The MEseNchymal coviD-19 Trial: a Pilot Study to Investigate Early Efficacy of MSCs in Adults With COVID-19 (NCT04537351)
Status:	Recruiting(Phase I,II)
Treatment:	Allogeneic mesenchymoangioblast-derived mesenchymal stem cells (MCA-derived MSCs)- (CYP-001)

Study Name:	ACT-20 in Patients With Severe COVID-19 Pneumonia (NCT04398303)
Status:	Not yet recruiting(Phase I,II)
Treatment:	Allogenic human umbilical derived mesenchymal stem cells (ACT-20-MSC), Allogenic human umbilical derived mesenchymal stem cells in conditioned media (ACT-20-CM)

Study Name:	Treatment of Severe COVID-19 Pneumonia With Allogeneic Mesenchymal Stromal Cells (COVID_MSV) (NCT04361942)
Status:	Recruiting (Phase II)
Treatment:	Mesenchymal Stromal Cells|

Study Name:	Repair of Acute Respiratory Distress Syndrome by Stromal Cell Administration (REALIST) (COVID-19) (NCT03042143)
Status:	Recruiting(Phase I,II)
Treatment:	Human umbilical cord derived CD362 enriched MSCs

Study Name:	Umbilical Cord(UC)-Derived Mesenchymal Stem Cells(MSCs) Treatment for the 2019-novel Coronavirus(nCOV) Pneumonia (NCT04269525)
Status:	Recruiting(Phase II)
Treatment:	UC-MSCs

Study Name:	Mesenchymal Stromal Cell Therapy For The Treatment Of Acute Respiratory Distress Syndrome (NCT04447833)
Status:	Active, not recruiting(Phase I)
Treatment:	Mesenchymal Stromal Stem Cells - KI-MSC-PL-205

Study Name:	Efficacy of Intravenous Infusions of Stem Cells in the Treatment of COVID-19 Patients (NCT04437823)
Status:	Recruiting(Phase II)
Treatment:	Intravenous Infusions of Stem Cells

Study Name:	Safety and Efficiency of Method of Exosome Inhalation in COVID-19 Associated Pneumonia (NCT04602442)
Status:	Enrolling by invitation(Phase II)
Treatment:	Exosome inhalation (EXO 1 inhalation)| (EXO 2 inhalation)

Study Name:	Evaluation of Safety and Efficiency of Method of Exosome Inhalation in SARS-CoV-2 Associated Pneumonia. (NCT04491240)
Status:	Completed(Phase I,II)
Treatment:	Exosome inhalation (EXO 1 inhalation)| (EXO 2 inhalation)

Study Name:	Cell Therapy Using Umbilical Cord-derived Mesenchymal Stromal Cells in SARS-CoV-2-related ARDS (NCT04333368)
Status:	Active, not recruiting(Phase I,II)
Treatment:	Umbilical cord Wharton's jelly-derived human Mesenchymal Stromal Cells|

Study Name:	MSCs in COVID-19 ARDS (NCT04371393)
Status:	Active, not recruiting (Phase III)
Treatment:	Expanded mesenchymal stromal cells derived from the bone marrow (Remestemcel-L)

Study Name:	Study of Descartes-30 in Acute Respiratory Distress Syndrome (NCT04524962)
Status:	Recruiting(Phase I,II)
Treatment:	RNA-engineered off-the-shelf allogeneic mesenchymal stem cell MSC (Descartes 30)

Study Name:	Multiple Dosing of Mesenchymal Stromal Cells in Patients With ARDS (COVID-19) (NCT04466098)
Status:	Recruiting(Phase II)
Treatment:	Mesenchymal stromal cells

Study Name:	Cellular Immuno-Therapy for COVID-19 Acute Respiratory Distress Syndrome - Vanguard (NCT04400032)
Status:	Recruiting(Phase I)
Treatment:	Mesenchymal Stromal Cells

Study Name:	A Pilot Clinical Study on Inhalation of Mesenchymal Stem Cells Exosomes Treating Severe Novel Coronavirus Pneumonia (NCT04276987)
Status:	Completed(Phase I)
Treatment:	MSCs-derived exosomes

Study Name:	Mesenchymal Stem Cells for the Treatment of Various Chronic and Acute Conditions (NCT04684602)
Status:	Recruiting(Phase I,II)
Treatment:	Human umbilical chord tissue-derived MSC (PrimePro™/ PrimeMSK™)

*data from ClinicalTrials.gov searched on March 6th 2021.

Future directions

Other clinical trials also accentuate the significance and safety of MSCs usage in the treatment of COVID-19 patients with no reporting of any serious adverse events related to MSCs (92-100), although there is a necessity for large randomized multicenter clinical trials in order to determine precise therapeutic potentials of MSC in COVID-19-induced disease (92,93,97). It is also very important to emphasize that MSCs should not be administered in the early period of viral infection because inflammation is very pertinent and advantageous to combat viral infection. “Physiological inflammation” which is very important for control of virus infection and replication, can be abrogated by too much immunosuppression that is induced by inadequate use of MSCs in terms of the time of MSCs administration and their dose (101-104). However, there is the urge to investigate the safety of using human MSCs in long period follow- up. It is well- known that MSCs exhibit potential risks of adverse events during MSC transplantation (105) in CVDs (cardiovascular disease), neurological disease, GVHD (graft- versus host disease), and orthopedics, such as pro-tumorigenic effect, perturbed differentiation capacity, short surviving after implantation, not so amazing improvements, immune response and infection-related mortality (106). Some animal studies reported that MSCs therapy for CVDs can exhibit some adverse events such as proarrhythmic (107) and tumorigenic ability in heart tissue (108) as well as the ability to differentiate into unwanted tissue type (109). Although many studies suggest the usage of MSCs as a treatment in orthopedics, it is important to note that MSCs are able to take part in ectopically forming bone tissue in non-bone tissues, which is better known as heterotopic ossification (HO) (110). Although MSCs are cells with doubtful- advantage in numerous animal studies that

showed MSCs promoted tumor growth and metastasis (111,112), or even suppressed their growth (112), MSCs should be used with caution in clinical studies.

CONCLUSION

All of the effects from MSCs in regards to immune system, regeneration, neoangiogenesis, antiviral capacity, ubiquitous presence in almost all postnatal tissues and ease of transplantation make MSCs potentially excellent choice against Covid-19. However, MSCs therapy is a double edged sword, and if not applied correctly and responsibly can worsen clinical condition of patients and lead to adverse effect. So, in spite the data from small number of completed clinical trials that displayed MSCs therapy as safe and beneficial and on the other hand, dozens of ongoing clinical trials, final conclusion is yet to be brought.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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DYSLIPIDEMIA IS A MAJOR SIDE EFFECT OF LONG-TERM ANTIRETROVIRAL THERAPY

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ABSTRACT

The aim of this study was to investigate the impact of different antiretroviral therapy on the lipid status of HIV patients with emphasis on modern-generation drugs. A cross-sectional study was conducted at Clinic for Infectious Diseases at the University Clinical Center Kragujevac and included forty-six patients with HIV infection on antiretroviral therapy for a minimum of twelve months. Lipid status parameters were analyzed in relation to the length of administration and the type of antiretroviral therapy used (integrase inhibitors or other antiretroviral therapy groups). The average duration of antiretroviral therapy intake ± standard deviation was 5.59 ± 3.649. Statistically significant higher values of low-density lipoprotein cholesterol were recorded after six years of antiretroviral therapy that does not belong to the group of integrase inhibitors compared to a period of less than three years ($p<0.05$). After six years of the administration of all groups of antiretroviral therapy, low-density lipoprotein cholesterol and total cholesterol values increase significantly compared to all other groups ($p<0,01$ and $p<0,05$, respectively). Patients on integrase inhibitors therapy compared to other antiretroviral therapy groups, show statistically significant higher total cholesterol values ($p<0,05$). Although low-density lipoprotein cholesterol values show a tendency to increase over time in both (integrase inhibitors and other antiretroviral therapy) groups, they do not differ, which means that integrase inhibitors do not have a greater impact on low-density lipoprotein cholesterol growth. Despite the use of modern-generation antiretroviral therapy, dyslipidemia is present in a significant percentage of HIV patients.

Keywords: *Dyslipidemia, HIV, antiretroviral therapy, INSTI.*

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INTRODUCTION

HIV continues to be a major global public health issue. In 2021, 650 000 people died from HIV-related causes and 1.5 million people acquired HIV. There were an estimated 38.4 million people living with HIV at the end of 2021 (1). Antiretroviral therapy (ART) is able to control HIV in most patients but not to cure the disease. Therefore, HIV remains in the reservoirs with potentially harmful effects and ART needs to be continuously given. Modern ART has significantly extended the life expectancy of those suffering from HIV infection and improved the quality of life. However, it has been observed that HIV-infected patients suffer from comorbidities such as cardiovascular disease, kidney disease, diabetes, and liver metabolic diseases at a much earlier age than the general population with possible deleterious consequences such as neurocognitive decline, frailty and multimorbidity.

First, a number of conditions, such as personal factors, family origin, age, and gender affect the level of lipid parameters. Studies have shown that HIV infection itself increases the risk of metabolic disorders independently of ART. Abnormal lipid levels have been reported early in ART-naive persons living with HIV (PLHIV) and are associated with the presence of acute infection. In contrast, other studies have shown that HIV infection and ART are equally represented in the development of metabolic disorders, which contributes to the development of non-AIDS comorbidities (2, 3). ART can induce raised levels of total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), and triglycerides (TG), as well as variable effects on high-density lipoprotein cholesterol (HDL-C) levels (4, 5, 6).

The first generation of ART, such as nucleoside analog reverse transcriptase inhibitors (NRTIs) and protease inhibitors (PIs) often caused dyslipidemia with metabolic consequences. The replacement of these molecules by newer ones, allowed to minimize the metabolic adverse effects of ART. But, contemporary ART as integrase inhibitors (INSTI) was recently also associated with weight and fat gain and possibly impaired glucose metabolism (7).

The mechanisms involved in causing metabolic disorders are poorly understood. Besides ART usage, HIV is still present in tissue reservoirs and has metabolic implications. Also, PLHIV receive several ART at a given time and have been often long-term exposure to a number of first-generation ART, with possible residual effects. ART adverse effects differ according to the class but also to the individual drug.

In addition, INSTI is at present the most used ART class, recommended in ART-naïve and ART-experienced PLHIV so the main goal is to understand the mechanisms involved in the INSTI effects on lipid profile.

MATERIALS AND METHODS

Our study was conducted in the Clinic for Infectious Diseases at the University Clinical Center Kragujevac. The study was approved by the Institutional Ethical Committee and it was in compliance with Helsinki Declaration.

The study was designed as cross-sectional and it included forty-six patients with proven HIV infection on ART for a minimum of twelve months who were treated at the Clinic for Infectious Diseases. Study data were collected from the outpatient clinic histories. The following variables were examined: age, gender, number of CD4 cells at the time of disease detection, viral load, type of ART, and length of ART usage. Laboratory values were obtained during routine outpatient examinations, from the blood sample, by standard laboratory tests. Biochemical parameters of lipid status - TC, TG, LDL-C and HDL-C were measured and presented in mmol per liter. Furthermore, the patients were divided into three groups in relation to the length of ART administration. The first group consisted of patients who used ART for less than three years, the second group of patients who took ART between three and six years, and the third group of patients who used ART for more than 6 years. Lipid status parameters were compared between these three groups of patients regardless of the type of ART therapy applied. After that, all the patients were divided into two groups depending on the type of ART therapy used and lipid status parameters were compared between groups. The first group included patients who used some of the drugs from the INSTI group. The second group included patients who used drugs from the other ART groups. Separately, the same comparison of lipid status parameters according to the length of ART use was conducted in patients who used only drugs from non-INSTI groups.

The software used for the analyses was SPSS version 26 (SPSS Inc, Chicago, IL). The study data were tested by descriptive statistics. Parameters of lipid status were presented by mean ± standard error. The other continuous variables were presented by mean ± standard deviation. Also, categorical variables were depicted with percentages.

The continuous variables were compared using the Independent samples t-test, One-way ANOVA or Mann-Whitney U test, and Wilcoxon's rank sum depending on the normality of distribution. P value was significant if less than 0.05.

RESULTS

Our study included forty-six PLHIV on ART. Out of them, 80.4% were men and 19.6% were women, while the mean age ± standard deviation was 41.85 ± 10.172. In relation to the initial CD lymphocyte count, the criteria for AIDS (CD4 lymphocyte count less than 200/mm^3) was fulfilled by 30.4% of patients, while the criteria for late presenters (CD4 lymphocyte count less than 350/mm3) were fulfilled by 54.3%. Viral load was determined in relation to the number of HIV copies detected with polymerase chain reaction

(PCR) in serum. Negative PCR was detected in 89.1% of patients, 40-100 copies per mm^3 of blood were detected in 6.5% and 400-500 copies were detected in 4.3%.

The average duration of ART intake ± standard deviation was 5.59 ± 3.649. There were 35.6% of patients who used ART for less than three years, 26.7% between three and six years, and 37.8% for more than 6 years. According to the type of applied ART, drugs from the INSTI group were used by 58.7% of patients (raltegravir frequency was 19.6% and lamivudine/abacavir/dolutegravir was 39.1%), and drugs from other ART groups were used by 41.3% of patients. Within the non-INSTI group, the frequency of tenofovir/emtricitabine was 26.1%, emtricatibine/tenofovir/rilpivirine was 30.4%, lamivudine-abacavir was 4.3%, darunavir/cobicistat was 8.7% and efavirenz was 2.2%.

In the group of patients who used only drugs from the non-INSTI group, statistically significant higher LDL-C values were observed after six years of drugs use compared to a period of fewer than three years (*Figure 1*). When we look at the lipogram parameters after six years of the administration of all groups of ART (INSTI and non-INSTI), LDL-C and TC values increase significantly compared to all other groups (*Figure 2*). Group of patients on INSTI therapy compared to other ART groups (non-INSTI), show statistically significant higher TC values in the INSTI group. Although LDL-C values show a tendency to increase over time in both groups, they do not differ, which means that INSTI does not have a greater impact on LDL-C growth compared to other ART therapy (*Figure 3*).

Other parameters of lipid status do not show significant differences during the years of follow-up.

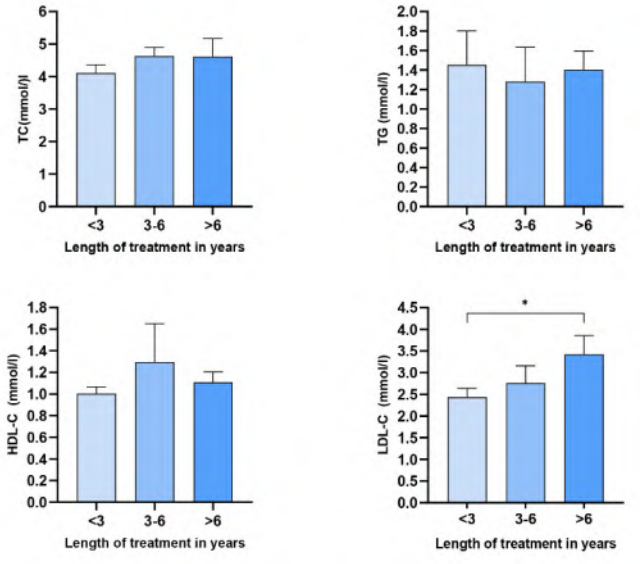

Figure 1. Values of lipid status parameters in three groups of patients according to the length of treatment with other (non-INSTI) ART. The values are presented as mean ± standard error of the mean (SEM), *denotes a significant difference p<0.05.

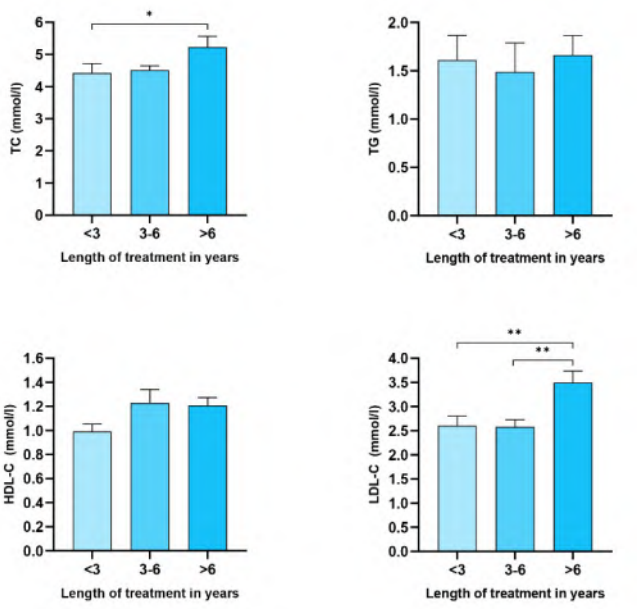

Figure 2. Values of lipid status parameters in three groups of patients according to the length of all ART administration (INSTI and non-INSTI group). The values are presented as mean ± standard error of the mean (SEM), *denotes a significant difference p<0.05, **denotes a significant difference p<0.01.

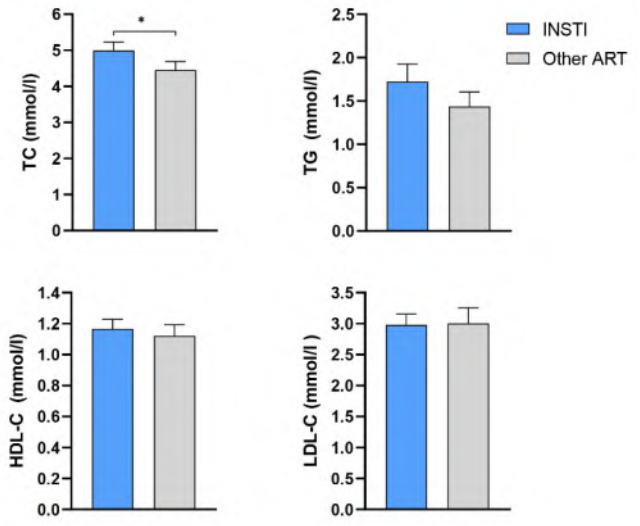

Figure 3. Values of lipid status parameters in relation to the type of ART applied. The values are presented as mean ± standard error of the mean (SEM), *denotes a significant difference p<0.05.

DISCUSSION

The occurrence of lipid disorders in people living with HIV is multifactorial. Previously, when ART was not available as it is today, various disorders of lipid metabolism were recorded in untreated patients (8).

It is considered that high viremia and low CD4 T lymphocytes have a significant influence on the occurrence of dyslipidemia in untreated persons with HIV (9). HIV interferes with the role of gut-associated lymphoid tissue, GALT because it depletes the residual Th 17 lymphocytes that are involved in the homeostasis of the intestinal epithelium. Therefore, microbial translocation is thought to be the main mechanism for immune activation and chronic inflammation (10). As a consequence of the direct effect of the virus, endothelial dysfunction occurs, which is characterized by a decrease in the anti-inflammatory and antithrombotic properties of the endothelium, an increase in the permeability of the endothelium, proinflammatory cytokines, and the expression of adhesion molecules. Due to the increased permeability of the endothelium and the transmigration of leukocytes, infiltrations rich in LDL-C occur, which represent the initial changes in arteriosclerosis, a process that is responsible for the development of cardiovascular comorbidities (3, 11).

Today, in the era of modern ART, the initiation of therapy occurs at the time of diagnosis, so lipid profile data in untreated patients are more difficult to obtain today. Determining the effect of a specific ART on the lipid profile is difficult because the therapy includes several drugs, and different classes of ART and different drugs within the class have a different effects on the lipid profile. In general, hyperlipidemia ranges from 28% to 80% according to different studies, which is consistent with the data in our research (12).

Initiation of ART treatment results in long-term viral suppression, reduction of inflammation, and immune reconstitution. However, it was found that the levels of cytokines in PLHIV on ART are similar to those of healthy controls, but who is older between 4 and 12 years, which indicates premature aging and the development of comorbidities (13).

The results of our research showed that the values of LDL-C and TC increase depending on the length of ART administration, which indicates its cumulative effect and the impact of ART on the occurrence of comorbidities. Traditionally, dyslipidemia was previously mostly associated with older drugs from the group of protease inhibitors, which were not included in our research, while only Darunavir was represented in 8.7% of respondents. Also, from the NNRTI group, only one patient recorded the presence of Efavirenz, while from this group, Rilpivirine was the most common. When we analyzed lipid profile values in the group of patients who did not have integrase inhibitors, the results showed that LDL-C values increased during the follow-up period. On the other hand, TC stood out in the INSTI group, as a parameter whose values increased over time, while INSTI did not show a negative impact on other lipid parameters. However, we cannot exclude an effect of previous therapies which may influence our results.

Examination of dyslipidemia in different classes of ART was a specifical subject of the RESPOND study on 4577 patients. Their results showed that dyslipidemia was less common with INSTI than with protease inhibitor. Compared with dolutegravir, dyslipidemia was more common with elvitegravir/cobicistat and raltegravir, but less common with rilpivirine (14).

It has already been described the association of INSTI, but also tenofovir-alafenamide (TAF), with weight gain, being higher compared to older groups of drugs (about 1 kg more in the first year) (15). Also, recent research has shown an increase in the risk of progression of steatosis in INSTI and TAF (16).

It is known that modern ART is much more potent than older groups of drugs and that it strongly suppresses viremia. Despite this, however, low levels of inflammation occur, affecting cholesterol transport and inducing pro-inflammatory changes in lipids, which lead to the development of arteriosclerosis and the occurrence of cardiovascular comorbidities, as well as increased mortality (12, 17).

CONCLUSIONS

Our results show that despite the use of modern-generation ART, dyslipidemia is present in a significant percentage of our patients. Dyslipidemia is involved in the development of the metabolic syndrome and represents a risk for the development of cardiovascular comorbidities, which today represent the most important cause of death in the group of non-AIDS diseases.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ANALYSIS OF RISK FACTORS FOR INAPPROPRIATE PRESCRIBING OF PSYCHOTROPIC DRUGS IN PRIMARY HEALTH CARE IN ELDERLY PATIENTS

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ABBREVIATIONS

STOPP -Screening Tool of Older Person's potentially inappropriate Prescriptions
START - Screening Tool to Alert doctors to the Right Treatment
WHO - World Health Organization;
PIM - Potentially Inappropriate Medicines;
PPO - Potential Prescription Omissions;
CNS – Central Nervosus System;
IPD - irrational prescribing of drugs;
IPPD - inappropriate prescribing of psychotropic drugs;
RSD - Serbian dinar;
SSRI - selective serotonin reuptake inhibitors;
SNRI - selective serotonin and norepinephrine reuptake inhibitors;
sGP *(general practitioner)* - selected general practitioner;
PSR *(pharmaceutical sales representative)* - expert associate of a pharmaceutical company.

ABSTRACT

Older people are at risk of inappropriate drug prescribing because pharmacodynamics and pharmacokinetics, and consequently the efficacy and safety of drugs, change after patient's age. The aim of study is to identify major significant risk factors for Potentially Inappropriate Medicines (PIM) of psychotropic drugs and Potential Prescription Omissions (PPO) of psychotropic drugs in population of patients over 65 years of age with associated pathological conditions according to Screening Tool of Older Person's potentially inappropriate Prescriptions/Screening Tool to Alert doctors to the Right Treatment (STOPP/START) criteria. The study was designed as a cross-sectional study involving 492 patients and 9 selected general practitioners. It was conducted in period from May 2020 to December 2021, after receiving decision from Ethics Committee of HC Kragujevac. 492 patients, mean age 71.77 ± 5.95, with 62.2% women, participated in the study. 164 PIMs were identified in 139 patients (28.2%). The most common were: use of benzodiazepines over 4 weeks (43.9%) with simultaneous use of different groups of antidepressants (20.3%). Patients with more than two psychotropic drugs have a higher risk for PIM [adjusted OR 2.83, 95% CI (1.98 - 4.140], p <0.001. 439 PPOs were also identified in 270 patients (54.8%). Risk factors for PPO are: age, number of illnesses, total number of medications, number patients, depression presence, patient's place of residence, cigarettes usage and monthly income level. STOPP/START criteria can have a major impact in recognizing inadequate prescribing of psychotropic drugs at patients over 65 years of age. Patients who use benzodiazepines more than four weeks and / or antidepressants may be at increased risk of PIM psychotropic drugs. The total number of drugs and presence of symptoms of depression bring higher risk of PPO psychotropic drugs.

Keywords: *STOPP/START, psychotropic drugs, older population.*

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INTRODUCTION

Age of population is an important health factor that is of great importance for analysis of the biological structure of population and is determined by ratio of older population in the total population in a given territory. The age of 65 years and older is considered as social age limit in modern living conditions[1,2]. Due to the intensive aging of the population nowadays, older population is emerging as biggest drug users, primarily due to the significantly higher frequency of various diseases at that age. Often, these patients suffer from several associated illnesses, which imposes need for simultaneous usage of bigger number of drugs at same time, whether it is causal or symptomatic treatment. Older people are often treated in several different health care institutions, which belong to different levels of health care, from primary to tertiary. Every visit to another health institution creates an additional risk for irrational use of drugs, due to lack of information related to history of disease coming from different sources and/or unsatisfactory communication between health care professionals and between health professionals and patients[3].

Aging leads to a decrease in neurons density of central nervous system (CNS). It is estimated that people over the age of 80 lose about 30% of brain mass, primarily gray mass, which is contributed by atherosclerosis, which leads to impaired brain function. Production of important neurotransmitters, including catecholamine, serotonin and acetylcholine, is reduced, which is associated with changes in mood, memory and motor functions[4]. The older population is particulary sensitive to substances that have an effect on the CNS. Therefore, they need to adjust doses of psychotropic drugs (benzodiazepines, barbiturates, antidepressants). Even drugs that do not have side effects on CNS, these effects are more noticeable at people over 65 years of age[5].

The significance of IPD problem older population is reflected in its high prevalence ranging from about 15% to almost 80% depending on study site (country, region, primary, secondary or tertiary level of health care) and overall methodological approach. In the examination (especially the type of instrument by which the IPD was identified)[6,7].

The aim of this research is to determine significant risk factors for potentially inappropriate prescribing of psychotropic drugs in a population of patients over 65 years of age with associated pathological conditions who are on an outpatient treatment regimen, using explicit STOPP START criteria[8] from 2015.

MATERIAL AND METHOD

Study population

The research was conducted on a sample of 492 consecutively selected, chronically ill patients aged ≥ 65 years, both sexes, with different sociodemographic and clinical characteristics, who receive health services at the expense of the compulsory health insurance fund.

Research was conducted in five out of nine general practice institutions that operate within the General Medicine Service of the Primary Health Care Center Kragujevac, in the period May 2020 - December 2021. It was conducted after the approval by the Ethics Committee of the Kragujevac Health Center. The research was conducted in compliance with the principles of the Declaration of Helsinki and in accordance with the principles of Good Clinical Practice. In order to collect the highest quality data, an unstructured questionnaire was prepared for patients or their caregivers and their Selected physicians (sGP). The research included nine selected general practitioners.

Study design

The design of the study is a cross-sectional study, in which they are with the help of STOPP and START criteria and sociodemographic questionnaire identified risk factors for Potentially Inappropriate Medicines (PIM) of psychotropic drugs and Potential Prescription Omissions (PPO) psychotropic drugs in patients over 65 years of age.

Inclusion and exclusion criteria

In order to collect as many patients as possible who have inappropriately prescribed psychotropic drugs in their therapy, following criteria for inclusion in the study will be respected when selecting study participents:

- patients on outpatient treatment are in primary health care;
- have at least two chronic diseases that require daily use of drugs, to use at least two prescription drugs every day;
- they have been prescribed the same prescription drugs in the last three months, and that they have received dated and signed informed consent for participation and testing.

Excluding criteria:

- patients under 65 years of age,
- patients who have not seen a doctor in the last 6 months,
- patients who died during the recruitment period,
- terminally or seriously ill patients who receive health care through the home treatment service,
- patients hospitalized during the research period, patients with malignant diseases who underwent chemotherapy and / or radiotherapy,
- patients with incomplete documentation,
- patients who are already involved in another study and patients who are treated on their own initiative in private health care institutions.

Calculation of the sample size

The required sample size was calculated using G* Power software[9] calculation of the sample size is based on the expected difference between subjects who have IPPD, ie those who do not have risk, in the prevalence of exposure to main risk factors for PIP and PMP recorded in previous studies.[10] Taking into account recently conducted studies in this field in the region of the world, 492 respondents were needed to conduct the research in terms of the minimum number of participants for this cross-sectional study.

Procession of the statistical data

The IBM SPSS Statistics 23 software package was used for statistical data processing. Frequencies and percentages are shown for category variables. Mean values and standard deviation are shown for continuous variables that follow the normal distribution, and the median and IQR are shown for continuous variables that do not follow the normal distributionContinuous variables were compared between groups by the Mann-Whitney-U test, while for the categorical ones the chi-square or Fisher's exact test was used. Univariate and multivariate logistic regression analysis was performed to identify significant predictors for the presence of START and STOPP criteria. Raw as well as adjusted odds ratio (OR) values are shown along with a 95% confidence interval.

RESULTS

Sociodemographic characteristics

Out of 515 respondents, 492 respondents agreed to participate in the study (96%). Socio-demographic characteristics of the respondents are shown in Table 1. The average age of the respondents was 71.77 ± 5.95, with 62.2% of women participating in the study. The number of diagnoses established in the study was 914. At least two psychotropic drugs were used in 240 patients (48.8%). The average number of prescribed drugs per patient was 4.37 ± 2.234.

Potentially Inappropriate Medicines PIM psychotropic drugs

According to the STOPP criteria, 164 PIM of psychotropic drugs were identified in 139 patients (28.2%). Nine out of 14 STOPP criteria were identified as inappropriate prescribing in this study. The most common use of benzodiazepines over 4 weeks (43.9%) and concurrent use of different groups of antidepressants (20.3%). 69.5% of PIM of psychotropic drugs were associated with four diagnoses: anxiety, depression, heart failure, and arterial hypertension. Using multivariate logistic regression, we identified independent risk factors for PIM of psychotropic drugs in our study (Table 2). Patients with more than 5 prescribed medications are at higher risk for PIM of psychotropic medications. A statistically significant higher risk for PIM was identified in patients diagnosed with depression [adjusted OR 18.13, 95% CI (3.36-97.70)], p=0.001. The total number of diseases shows a statistically significant risk for PIM of psychotropic drugs [adjusted OR 8.80, 95% CI (1.91-7.57)], p=0.001. Patients number of sGP may be a potential risk factor for PIM of psychotropic drugs [adjusted OR 1.03, 95% SI (1.00-1.05)], p=0.027. Patient's place of residence [adjusted OR 2.73, 95% SI (1.05-7.078)], p=0.038 and bad life habits [adjusted OR 0.098, 95% SI (0.017-0.560)], p=0.009 such as cigarettes usage more than one pack per day, may be risk factors for PIM of psychotropic drugs. Visits number of PSR may be a risk factor for PIM of psychotropic drugs, [adjusted OR 0.069, 95% CI (0.020-0.232)], p=0.001.

The influence of several risk factors on the probability that PIM of psychotropic drugs occurred in the study is explained by the values of coefficient between 59.6% (Cox and Snell) and 79.5% (Negelkerke), which confirm the IPPD.

Potential Prescription Omissions (PPO) psychotropic drugs

According to the START criteria, 439 psychotropic PPO were identified in 270 patients (54.8%). Four out of 6 START criteria were identified as a potential failure of prescribing psychotropic drugs. The most common omissions in prescribing psychotropic drugs were related to the use of antipsychotics [adjusted OR 4.04, 95% CI (1.73-9.47)], p=0.001 and anxiolytics [adjusted OR 0.303, 95% CI (0.140-0.657)], p=0.002. Using multivariate logistic regression, we identified independent risk factors for PPO of psychotropic drugs in our study (Table 3). The number of diagnostic procedures performed in the last year may be a risk factor for PPO of psychotropic drugs [adjusted OR 1,262, 95% CI (1,036-1,537)], p = 0.002. Place of residence [rural life adjusted OR 11.33, 95% CI (2,838-45,273], p=0.001, and single life [adjusted OR 0.27, 95% CI (0.14-0.51], p=0.001, can be potential risk factors for PPO of psychotropic drugs.

The influence of several risk factors on the probability that PPO psychotropic drugs occurred in the research is explained by the values of coefficient between 41.6% (Cox and Snell) and 56.5% (Negelkerke), which confirm the IPPD.

DISCUSSION

The emergence of inappropriate prescribing of drugs is present today at all levels of health care in countries with different levels of socio-economic development, and the factors that affect it are monitored and examined over the years. According to results of literature search so far, this is one of the first researches that deals with the inappropriate prescribing of psychotropic drugs.

The results of the research indicate a high rate of IPPD. Excessive use of benzodiazepines for more than four weeks in patients over 65 years of age is present in Switzerland[11], United Kingdom[12] and Australia[13]. The reason for frequent use may be the problem that benzodiazepines do not have a restrictive diagnosis when prescribing, but also availability in pharmacies. In our study, the place of getting the therapy in

terms of private or public pharmacies, did not show statistical significance (p>0.005). Concurrent use of antidepressants and benzodiazepines coincident with use in Asian countries[14.] Simultaneous application is most often due to overlapping reports at the primary and tertiary levels health care. According to STOPP criteria, antidepressants are contraindicated in patients with heart disease and dementia[15,16]. The use of antidepressants of different groups (SSRI/SNRI) can lead to worsening of the symptoms of the underlying diseases (dementia, heart failure). A statistically significant higher risk for PIM in patients diagnosed with depression [adjusted OR 18,13, 95% SI (3.36 - 97.70)], p = 0.001, indicates that sGP should pay more attention to this phenomenon. The number of patients of sGP, place of residence of patients proved to be risk factors for PIM outcomes of psychotropic drugs. The obtained results coincide with the results from Serbia[17], which indicate that the avoidance of large poly-pharmaceuticals and the application of non - pharmacological measures can reduce the risk of PIM. Reducing the exposure of sGP to promotion material of PSR can also reduce the risk of PIM[18,19].

With this research, the potential prescribing failure of psychotropic drugs is reflected in the omission of antipsychotic and anxiolytic therapy. This phenomenon can be partly explained by the abuse of benzodiazepines[20] so the symptoms of these psychotic disorders are often masked by the use of benzodiazepines. A study in Serbia from 2018 showed that benzodiazepines from the group of anxiolytics are used in a high percentage in our population, which coincides with the results of our research. On the other hand, benzodiazepines, as effective and almost irreplaceable drugs in some indication areas, are exposed to a negative campaign by the pharmaceutical industry for pharmacoeconomic reasons[21], which is also confirmed by this study.

The shortcomings of this research relate to the limitations of "face to face" survey due to the current COVID-19 pandemic in our country, which has affected population of patients over 65 years old the most. Prescribing therapy with sGP was done in person, but also by phone. Often, the data from the socio-demographic questionnaire regarding patient were obtained from family members/guardians who came to the outpatient clinics of the Kragujevac Medical Center mainly to extend the therapy or to receive instructions for emergency specialist-consultative services.

Table 1. Sociodemographic characteristics of the study population

Characteristics of the study population	Total (n=492) n (%); mean+SD; median (IQR)
Age	71.77±5.954; 70.0; (6.0)
Sex (female)	306 (62.2%); 186; (37.8)
Total number of prescribed medications	4.37±2.234; 4.0; (3.0)
Most common diagnoses	
arterial hypertension	254 (51.6%); 238; (48.4)
cardiac insufficiency	218 (44.3%); 274; (55.7)
depression	106 (21.5%); 386; (78.5)
type 1 diabetes mellitus	80 (16.3%); 412; (83.7)
benign prostatic hyperplasia	70 (14.2%); 422; (85.8)
asthma	26 (5.3%); 466; (94.7)
epilepsy	18 (3.7%); 474; (96.3)

Table 2. Risk factors associated with potentially inappropriate prescribing of psychotropic drugs according to STOPP/START criteria

STOPP criteria	Raw OR 95% (CI)		Adjusted OR 95% (CI)	
Risk factors				
Arterial hypertension	2.451 (1.705-3.522)	**0.000**	0.419 (0.141-1.245)	0.118
Cardiac insufficiency	0.604 (0.422-0.864)	**0.006**	1.296 (0.470-3.575)	0.617
Asthma	2.431 (1.036-5.701)	**0.041**	1.329 (0.231-7.644)	0.750
Depression	93.449 (22.711-384.515)	**0.000**	**18.138 (3.367-97.704)**	**0.001**
Number of diseases	2.332 (1.858-2.927)	**0.000**	**3.805 (1.911-7.578)**	**0.001**
Total number of drugs	1.391 (1.265-1.530)	**0.000**	1.031 (0.812-1.310)	0.801
Problems with the use of psychotropic drugs	24.554 (5.863-102.834)	**0.000**	**43.517 (5.453-347.267)**	**0.001**
Number of OTC sales	2.029 (1.536-2.680)	**0.000**	1.625 (0.892-2.959)	0.113
Number of dietary supplements	1.538 (1.218-1.942)	**0.002**	1.262 (0.689-2.309)	0.451

STOPP criteria	Raw OR 95% (CI)		Adjusted OR 95% (CI)	
Use of antidepressants	84.389 (20.501-347.370)	**0.000**	**52.810 (6.217-448.561)**	**0.000**
PSR visits				
once a week	Ref.			
two/more times a week	0.203 (0.120-0.343)	**0.000**	**0.069 (0.020-0.232)**	**0.001**
once every two weeks	0.063 (0.037-0.109)	**0.000**	0.508 (0.196-1.316)	0.163
Number of patient examinations in the last 12 months	1.388 (1.255-1.535)	**0.000**	1.307 (1.005-1.699)	0.046
Number of hospitalizations in the last 12 months	1.540 (1.248-1.900)	**0.000**	1.089 (0.736-1.612)	0.669
Number of performed diagnostic procedures	1.233 (1.111-1.369)	**0.000**	1.196 (0.930-1.539)	0.163
Years of service of the eGP	1.138 (1.104-1.173)	**0.000**	1.017 (0.926-1.116)	0.728
Number of patients of the sGP	1.004 (1.003-1.006)	**0.000**	**1.003 (1.000-1.005)**	**0.027**
Number of adverse reactions to psychotropic drugs	6.411 (2.222-18.500)	**0.001**	0.572 (0.029-11.313)	0.714
Age	1.031 (1.001-1.063)	**0.045**	**0.920 (0.861-0.982)**	**0.012**
Place of residence				
city	Ref.			
suburb	3.362 (2.241-5.044)	**0.000**	**2.738 (1.059-7.078)**	**0.038**
rural life	6.562 (3.299-13.054)	**0.000**	2.979 (0.577-15.377)	0.192
Education				
uneducated	Ref.			
primary school	0.541 (0.316-0.929)	**0.026**	0.823 (0.240-2.823)	0.756
High School	0.847 (0.489-1.467)	0.554	0.484 (0.096-2.443)	0.379
college	0.286 (0.082-0.998)	**0.050**	0.023 (0.000-2646.848)	0.525
Cigarette consumption				
non smoker	Ref.			
one pack a day	2.231 (1.537-2.504)	**0.000**	1.612 (0.493-5.266)	0.429
more than one pack	1.468 (0.916-2.353)	0.111	**0.098 (0.017-0.560)**	**0.009**
Alcohol consumption				
does not consume	Ref.			
one cup per day	2.530 (1.634-3.917)	**0.000**	2.824 (0.917-8.699)	0.071
more than one cup	3.053 (1.947-4.788)	**0.000**	2.870 (0.600-13.716)	0.187
Physical activity				
inactive	Ref.			
daily	0.976 (0.622-1.531)	0.914	**4.809 (1.370-16.882)**	**0.014**
once a week	1.174 (0.738-1.869)	0.498	2.167 (0.546-8.595)	0.271
several times a week	4.583 (2.254-9.318)	**0.000**	**7.203 (1.562-33.210)**	**0.011**
Diet				
less than three meals				
three meals	Ref.			
three meals and two	0.447 (0.301-0.663)	**0.000**	0.491 (0.172-1.400)	0.183
snacks	0.527 (0.313-0.888)	**0.016**	1.461 (0.330-6.470)	0.617
Monthly income				
5,000 - 10,000	Ref.			
11,000 -15,000	0.626 (0.396-0.991)	**0.046**	**0.372 (0.140-0.992)**	**0.048**
16,000 - 20,000	0.147 (0.086-0.253)	**0.000**	**0.065 (0.016-0.270)**	**0.000**
over 20,000 RSD	0.293 (0.100-0.861)	**0.026**	1.050 (0.158-6.993)	0.960

***p**-statistical significance

Table 3. Risk factors associated with potential failure to prescribe psychotropic drugs according to STOPP / START criteria

START criteria	Raw OR 95% (CI)		Adjusted OR 95% (CI)	
Risk factors				
Arrhythmia	0.478 (0.303-0.752)	**0.001**	0.756 (0.331-1.724)	0.505
COPD	0.242 (0.075-0.782)	**0.018**	**0.033 (0.005-0.207)**	**0.001**
Depression	3.929 (2.278-6.779)	**0.000**	1.507 (0.499-4.552)	0.467
Number of diseases	1.277 (1.060-1.537)	**0.010**	1.198 (0.787-1.826)	0.400
Total number of drugs	1.227 (1.120-1.344)	**0.000**	**1.458 (1.172-1.815)**	**0.001**
Number of OTC sales	1.757 (1.321-2.338)	**0.000**	1.514 (0.943-2.433)	0.086
Number of dietary supplements	1.445 (1.136-1.838)	**0.003**	1.328 (0.867-2.036)	0.192
Antipsychotics	1.866 (1.167-2.982)	**0.009**	**4.049 (1.730-9.476)**	**0.001**
Anxiolytics	0.578 (0.349-0.849)	**0.005**	**0.303 (0.140-0.657)**	**0.002**
PSR visits				
once a week	Ref.			
two/more times a week	0.238 (0.146-0.387)	**0.000**	1.689 (0.596-4.784)	0.324
once every two weeks	0.632 (0.390-1.026)	0.064	**0.342 (0.132-0.886)**	**0.027**
Number of patient examinations in the last 12 months	1.110 (1.016-1.214)	**0.021**	0.968 (0.766-1.225)	0.788
Number of hospitalizations in the last 12 months	1.355 (1.090-1.685)	**0.006**	1.104 (0.820-1.486)	0.514
Number of performed diagnostic procedures	1.174 (1.056-1.305)	**0.003**	**1.262 (1.036-1.537)**	**0.021**
Degree of professional development of the sGP				
general practitioner	Ref.			
specialist in general medicine	3.919 (2.211-6.945)	**0.000**	0.953 (0.205-4.440)	0.952
Number of patients of the sGP	1.003 (1.002-1.004)	**0.000**	1.002 (1.000-1.004)	0.091
Place of residence				
city	Ref.			
suburb	1.308 (0.878-1.947)	0.186	2.065 (1.021-4.177)	0.05
rural life	3.385 (1.636-7.000)	**0.001**	**11.334 (2.838-45.273)**	**0.001**
Community living				
marriage	Ref.			
alone	0.404 (0.275-0.593)	**0.000**	**0.276 (0.148-0.516)**	**0.001**
with friends	1.327 (0.263-6.698)	0.732	4.437 (0.043-452.717)	0.528
with relatives	0.442 (0.108-1.807)	0.256	0.070 (0.002-2.256)	0.148
Cigarette consumption				
non smoker	Ref.			
one pack a day	1.984 (1.301-3.025)	**0.001**	1.242 (0.535-2.883)	0.614
more than one pack	2.104 (1.277-3.466)	**0.004**	1.862 (0.663-5.233)	0.238
Alcohol consumption				
does not consume	Ref.			
one cup per day	2.824 (1.804-4.422)	**0.000**	2.094 (0.884-4.956)	0.093
more than one cup	2.527 (1.607-3.974)	**0.000**	1.478 (0.556-3.931)	0.433
Diet				
less than three meals	Ref.			
three meals	0.640 (0.431-0.951)	**0.027**	1.399 (0.663-2.953)	0.379
three meals and two snacks	1.219 (0.700-2.121)	0.484	**4.376 (1.540-12.430)**	**0.006**
Monthly income				
5,000 - 10,000	Ref.			
11,000 -15,000	0.603 (0.381-0.953)	**0.030**	0.774 (0.345-1.737)	0.535
16,000 - 20,000	0.977 (0.586-1.629)	0.928	**2.900 (1.203-6.993)**	**0.018**
over 20,000 RSD	0.488 (0.171-1.392)	0.180	1.426 (0.310-6.550)	0.648

START criteria	Raw OR 95% (CI)		Adjusted OR 95% (CI)	
Social activities				
parties	Ref.			
travel adventures	1.476 (0.789-2.784)	0.229	1.903 (0.702-5.158)	0.206
theatrical performances	0.278 (0.085-0.905)	**0.034**	**0.253 (0.044-1.457)**	0.124
sport matches	1.997 (1.085-3.678)	**0.026**	**0.709 (0.282-4.841)**	0.464
political activities	1.216 (0.633-2.336)	0.558	1.492 (0.460-4.841)	0.505

***p-** statistical significance

CONCLUSION

28.2% of patients with PIM and 54.8% of patients with PPO indicate a high rate of IPPD, which suggests that STOPP/START criteria may be useful in identifying inappropriate prescribing, improving current prescribing and dispensing of psychotropic drugs. The implementation of STOPP/START criteria in everyday practice would greatly improve the current regulatory policy. Pharmacists should also focus more on patients with over four drugs that suffer from anxiety, depression, heart failure and arterial hypertension, as these patients may be at higher risk for PIM. In addition, more frequent diagnostic procedures, adequate therapy for depression and anxiety disorders can reduce the risk of PPO psychotropic drugs. All of the above, requires greater synchronization of health care in the relationship medical specialist – sGP - pharmacist.

CONFLICTS OF INTEREST

There is no conflict of interest among the signed authors.

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ANTITUMOR ACTIVITY OF PALLADIUM(II) COMPLEXES ON DU-145 CELL LINE *IN VITRO*

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ABSTRACT

In the area of non-platinum complexes, various complexes containing gold, copper, ruthenium, and palladium have shown a strong cytotoxic effect on different cancer cell lines. The aim of our study was to examine the cytotoxicity of the Pd(II) complexes (C1-C5) and the corresponding ligands (L1-L5) on the DU-145 prostate cancer cell line. Also, due to its clinical application, the cytotoxicity of cisplatin has been examined. Our findings showed that C1- C5 complexes and cisplatin show dose-dependent and strong cytotoxic effects against the DU-145 cell line in vitro. Furthermore, the results demonstrated that early apoptosis was induced by all five Pd(II) complexes. Also, the results showed that complexes C1, C3, and C5 induced G0/G1 phase arrest on DU-145 cells. Pd(II) complex C2 induced S phase arrest, while C4 complex induced G2/M phase arrest on cancer cells. Additionally, all tested complexes significantly reduced the amount of antiapoptotic protein Bcl-2. Also, there was a significant increase in the concentration of proapoptotic Bax protein in DU-145 cells treated C1-C5 complexes. The results of our research demonstrated that Pd(II) complexes induced apoptosis via the mitochondrial pathway. Thus, it is crucial to further investigate the cytotoxicity of these Pd(II) complexes in vivo. Complex C2 might be a good candidate for a new generation of anticancer drugs.

Keywords: *antitumor activity, palladium(II) complexes, DU-145 cell line, in vitro*

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INTRODUCTION

Cisplatin is the most used drug in the treatment of various types of cancer (1, 2). Despite wide and successful therapeutic use, the side effects of cisplatin therapy are very serious (2, 3). Side effects include nephrotoxicity, hematological toxicity, gastrointestinal toxicity, neurotoxicity, cardiotoxicity, ototoxicity, and hepatotoxicity (2-4). It is also important to point out that drug resistance may occur after long-term cisplatin therapy (4, 5). In the area of non-platinum complexes, various complexes containing gold, copper, ruthenium, and palladium have shown a strong cytotoxic effect on different cancer cell lines (6-9). Also, many of these non-platinum complexes showed a high degree of selectivity which means they had a low effect on the viability of healthy cells *in vitro* (10-12). Therefore, transition metals represent the future in the treatment of different types of cisplatin-resistant cancer.

The most important mechanism for controlling cell proliferation is apoptosis (13, 14). As a result, the modern strategy in the approach to various cancer treatments is based on the induction of the apoptotic process (14, 15). Antiapoptotic Bcl-2 and proapoptotic Bax protein play a crucial role in apoptosis (15-17). Therefore, changes in the expression of the Bcl-2 and Bax proteins may lead to activation or inhibition of apoptosis.

Earlier studies demonstrated that Pd(II) complexes showed strong anticancer activity against different types of tumor cells (18-20). Studies have also revealed that Pd(II) complexes exhibited a strong cytotoxic effect by inducing apoptosis (18, 21, 22). Hence, our study aim was to examine the cytotoxicity of the Pd(II) complexes (C1-C5) and the corresponding ligands (L1-L5) on the DU-145 prostate cancer cell line. Moreover, we wanted to determine the mechanism of cell death that C1-C5 complexes induce in DU-145 tumor cells.

MATERIAL AND METHODS

MTT assay

The initial step in our study was to determine the cytotoxic effect of five Pd(II) complexes (C1-C5) and corresponding ligands (L1-L5) on human prostate cancer cell line DU-145 by MTT test (23). The cytotoxicity of cisplatin on cancer cells was also investigated. The tumor cells were harvested from the culture flasks during the exponential growth phase, counted and 5×10^3 cells/well were seeded into 96-well culture plates. In addition, cells were incubated in an atmosphere containing 5% CO_2 and at 37ºC for 24 hours and then treated with several concentrations (0.3, 1, 3, 10, 30, and 100 μM) of C1-C5 complexes, L1-L5 ligands, cisplatin, and with the fresh complete medium as a control. DU-145 cells were incubated at 37°C in an atmosphere containing 5% CO_2 and at absolute humidity for 24, 48, and 72 hours. After incubation, the medium was separated and MTT solution was added to each well. Next, the solution was gently removed and formazan crystals were dissolved in DMSO. Microtiter plates were shaken in the dark for 10 min and absorbance was measured at 595 nm with a multiplate reader (Zenyth 3100, Anthos Labtec Instruments, Austria). Experiments were performed in triplicates and repeated in three independent series. The percentage of viable cells was calculated by dividing the value of the readout absorbance in the wells that contained treated cells with the average absorbance value measured in the wells of untreated cells, and the ratio thus obtained was multiplied by 100.

% of the viable cells = ((absorbance of treated cell-absorbance of blank) / (absorbance of untreated cell-absorbance of blank)) * 100

The IC_{50} values (values that reduce the treated cells' viability by 50% relative to the control) were determined using Microsoft Office Excel 2010 via logarithm-transformed dose-response data, previously obtained by MTT assay.

Annexin V/PI assay

Apoptosis of DU-145 tumor cell line was estimated by Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Kit (BD Biosciences). DU-145 cancer cells were incubated with previously calculated IC_{50} values of Pd(II) complexes or with media alone (control) for 24h at 37°C in an atmosphere of 5% CO_2 and absolute humidity. Furthermore, DU-145 cells were trypsinized, washed in phosphate buffer saline (PBS), centrifuged, and resuspended in 100 μL of ice-cold binding buffer. Then, we stained cells with both 10 μL of Annexin V-FITC and 20 μL of PI, incubated for 15 min in the dark at room temperature, and to each tube, 400 μL of binding buffer was added. Samples were measured using flow cytometer Cytomics FC500 (Beckman Coulter). Also, obtained data were analyzed using FlowJo V10 Software. Measurements were presented as density plots of Annexin V-FITC and PI stainings.

Cell cycle analysis

The next action in our research was to analyze the potential effects of C1-C5 complexes on the cell cycle of DU-145 cells. Tumor cells were incubated with the IC_{50} concentrations of C1-C5 complexes and cisplatin or with media alone (control) for 24h at 37°C in an atmosphere of 5% CO2 and at the absolute humidity. Moreover, DU-145 cells were harvested, washed with PBS, and fixed with 70% ethanol at +4 °C. Cells were agglomerated and resuspended in 1 mL PBS containing RNAse A (500 μg/mL). Following an incubation period of 30 min at 37°C, tumor cells were treated with 5 μL PI (10 mg/mL PBS). The samples were evaluated after 15 min of incubation in the dark by a flow cytometer. The cell cycle distribution was defined using FlowJo V10 Software and the results were introduced as histograms.

Assessment of apoptosis

One of the main goals of our research was to examine the expression of the proapoptotic protein Bax, antiapoptotic protein Bcl-2, and the percentage of cells containing active

caspase-3. DU-145 cells were incubated for 24 h with IC_{50} concentration of C1-C5 complexes or in a complete cell culture medium (control). In addition, DU-145 cells were washed three times with ice-cold PBS, resuspended, fixed, and permeabilized (Fixation and Permeabilization Kit, eBioscience). For Bcl-2 staining, the cells were incubated with 1:1000 Bcl-2 fluorescein isothiocyanate (FITC) primary antibody (mhbcl01, Life technologies) for 15 min at room temperature. Additional staining included incubation of permeabilized DU-145 cells for 30 min with 1:1000 of primary antibodies for active-Bax (N20, sc-493; Santa Cruz Biotech Inc.) and cleaved caspase-3 (#9661, Cell signaling Technology). Also, cells had been washed with PBS and incubated with the 1:2000 secondary goat anti-rabbit IgG-FITC antibody (Ab6717-1, Abcam) for 30 minutes. Afterward, cells were washed in PBS and analyzed by flow cytometry. Fluorescence of at least 15000 events/sample had been measured using FC500 (Beckman Coulter). Fluorescence intensity was standardized using isotype-matched negative control antibodies. The mean fluorescence intensities for Bax and Bcl-2 (MFIs) were calculated as the ratio of raw mean channel fluorescence to isotype control levels, respectively, and represent the level of expression of these proteins. The cleaved caspase-3 concentrations were evaluated as the percentages of cells displaying the fluorescence.

Statistical analysis

The distributions of the obtained data were evaluated for normality using the Shapiro-Wilk test. The values of MTT and apoptotic protein assays were presented as mean ± standard deviation (SD). The values of annexin and cell cycle assays were presented as medians due to large standard deviations and the distribution of these data that was not normal. All experiments were performed in triplicates and three separate repetitions. Commercial SPSS version 20.0 for Windows was used for statistical analysis. Statistical evaluation was performed by Student's T-test for paired observations, or one-way ANOVA depending on data distribution. P values less than 0.05 were considered to indicate a statistically significant difference.

RESULTS

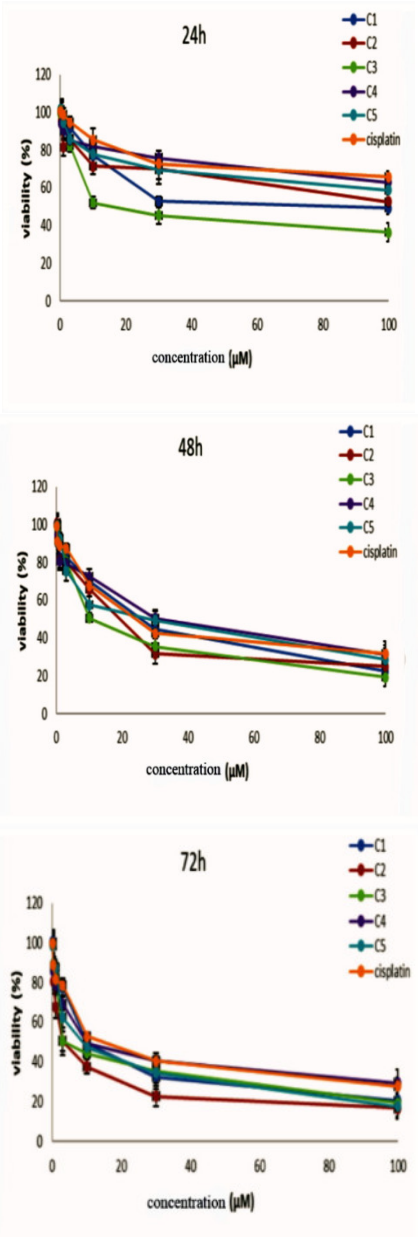
Cytotoxicity of Pd complexes

The antitumor effect of five Pd(II) complexes (C1-C5) and corresponding ligands L1-L5 was estimated on human prostate cancer cell line (DU-145) by MTT test after 24, 48, and 72h of treatment. Also, due to its clinical application, the cytotoxicity of cisplatin has been examined. Our findings showed that C1- C5 complexes and cisplatin show dose-dependent cytotoxic effects against the DU-145 cell line *in vitro* (Figure 1). The IC_{50} values for C1-C5 complexes, L1-L5, and cisplatin are presented in Table 1. Additionally, all five tested ligands L1-L5 showed weak cytotoxicity against the DU-145 cell line, exhibiting IC_{50} values of >200 μM. Also, both Pd(II) complexes and cisplatin showed high cytotoxicity against DU-145 cells after 72h of treatment. (Table 1).

Table 1. IC50 values for ligands L1-L5, Pd(II) complexes C1-C5 and cisplatin after 24, 48, and 72h drug exposure.

IC_{50} (μM)	DU-145		
	24h	48h	72h
L1	>200	>200	>200
L2	>200	>200	>200
L3	>200	>200	>200
L4	>200	>200	>200
L5	>200	>200	>200
C1	82,9	50,3	41,7
C2	98,7	47,8	26,21
C3	60,8	44,7	36,5
C4	134,9	59,1	47,6
C5	111,8	55,1	38,9
cisplatin	134,5	57,2	49,2

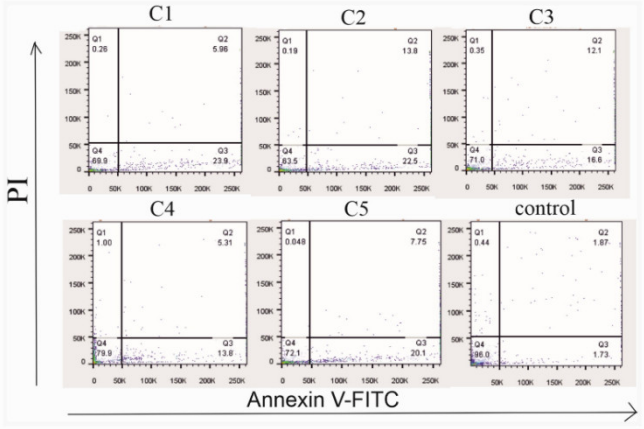
Figure 1. The effects of Pd(II) complexes (C1-C5) and ligands (L1-L5) on the viability of human prostate cancer cells DU-145.



Effects of Pd(II) complexes on apoptosis

The earlier described results of our research proved that all five (C1-C5) complexes showed a strong, dose-dependent antitumor effect against the DU-145 cell line. Thus, the following phase of our study was to investigate the type of DU-145 cells' death generated by these Pd(II) complexes. The findings demonstrated that early apoptosis was induced by all five Pd(II) complexes (Figure 2). In addition, a negligible percentage of the DU-145 cells were necrotic and in late apoptosis, while the rest of the non-viable cancer cells were in early apoptosis.

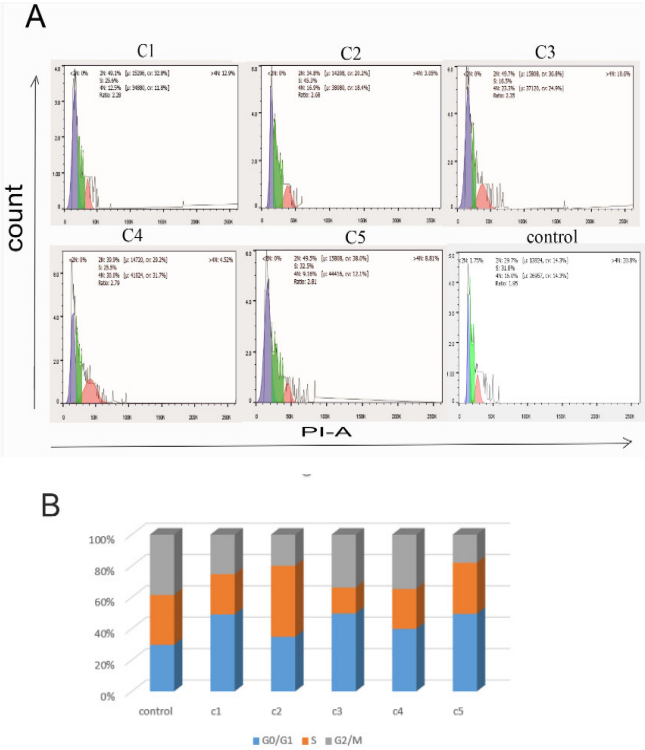
Figure 2. Pd(II) complexes (C1-C5) reduce the viability of treated DU-145 cells predominantly by induction of apoptosis



Effects of Pd(II) complexes on cell cycle of tumor cells

It is well-known that stimulation of the apoptotic process and/or cell cycle arrest may decrease the viability of tumor cells. In our study, the cell cycle was examined 24h after the treatment of DU-145 tumor cells with IC_{50} concentrations of C1-C5 complexes by flow cytometry in PI-stained cancer cells (Figure 3). Our results demonstrated that complexes C1, C3, and C5 induced G0/G1 phase arrest on DU-145 cells. Furthermore, Pd(II) complex C2 induced S phase arrest, while C4 complex induced G2/M phase arrest on cancer cells.

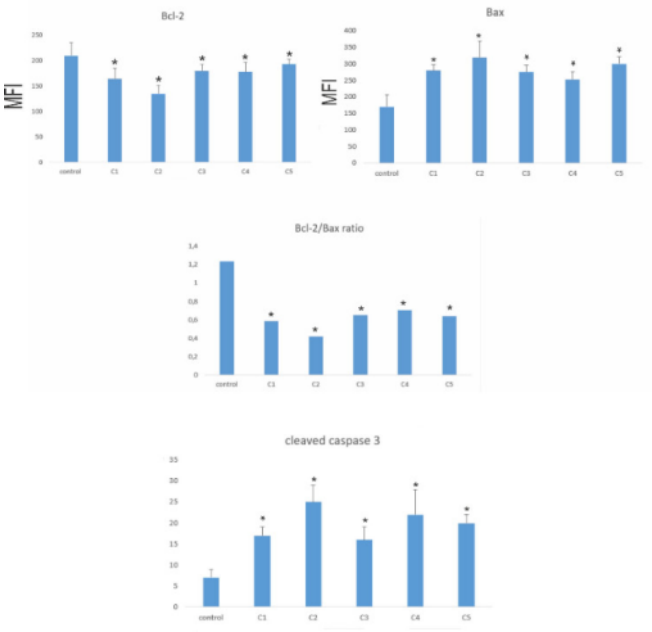
Figure 3. Pd(II) complexes (C2-C5) induce cell cycle arrest of treated DU-145 cells.



Pd(II) complexes induce apoptosis via the mitochondrial pathway

In the next step, we wanted to investigate whether Pd(II) complexes changed the cytoplasmic concentration of antiapoptotic protein Bcl-2 and pro-apoptotic protein Bax. Furthermore, we evaluated the activation of the caspase-3 in tumor cells treated with C1-C5 complexes. The findings undoubtedly showed that all tested complexes significantly reduced the amount of antiapoptotic protein Bcl-2 (Figure 4, $p<0,05$). Also, there was a significant increase in the concentration of proapoptotic Bax protein in DU-145 cells treated C1-C5 complexes (Figure 4, $p<0,05$). Importantly, the percentage of cells containing active caspase-3 was also increased in cancer cells treated by Pd(II) complexes (Figure 4). Consequently, C1-C5 complexes decreased the Bcl-2/Bax ratio (in comparison to control) which managed the activation of caspase-3 and induction of apoptosis via the mitochondrial pathway.

Figure 4. Pd(II) complexes C1-C5 induce apoptosis of human prostate cancer cells DU-145 via the mitochondrial pathway.



DISCUSSION

Metal complexes have become essential in medical research due to their physicochemical properties, their multiple oxidation states, and stereochemistry, making them appropriate candidates for future antitumor drugs (24, 25, 26). In our research, we synthesized five Pd(II) complexes and the focus was to explore the biological activity of complexes and corresponding ligands (L1-L5). Thus, we examined cytotoxicity and the mechanism of action against human prostate cancer cells DU-145. We can unquestionably conclude from the presented results that C1-C5 complexes showed high cytotoxic activity against the DU-145 tumor cell line. In addition, complex C2 showed higher cytotoxic activity than cisplatin on tumor cells. Furthermore, Pd(II) complexes C3 and C5 exhibited slightly lower IC_{50} values compared to cisplatin after 72 hours of treatment. Unlike C1-C5 complexes, the corresponding ligands L1-L5 exhibited very low antitumor activity on the DU-145 cells.

Similar to our results, Hernandez et al.'s study showed that five Pd(II) complexes had significant anticancer activity against DU-145 cells *in vitro* (27). Also, Carreira et al.'s study exhibited that Pd(II) complexes showed high cytotoxic activity against the human prostate cancer cell line DU-145 (28). Furthermore, Plutin et al.'s report demonstrated that some of the synthesized Pd(II)complexes showed potent antitumor activity on DU-145 cells (29). We can undoubtedly conclude that the results of our research agree with the results of the previously mentioned authors.

The apoptotic process is activated by two important signaling pathways: the intrinsic pathway, and the extrinsic pathway (30, 31). The intrinsic pathway is activated when an injury is inside the cell and the following stress activates the intrinsic pathway of apoptosis via mitochondria and the endoplasmic reticulum (30-32). The extrinsic pathway starts outside a cell when conditions in the extracellular environment determine that a cell must enter the process of apoptosis (32, 33). Our previously defined results showed that Pd(II) complexes (C1-C5) exhibited cytotoxicity against DU-145 cancer cells by induction of apoptosis. In addition, Joksimovic et al.'s research also showed that Pd(II) complexes induced apoptosis in cancer cell lines (34). Furthermore, Espino et al. and Keswani et al.'s researches showed that Pd(II) complexes induced apoptosis via the intrinsic (mitochondrial) pathway through an increase of the concentration of proapoptotic Bax protein associated with the activation of caspase-3 (35, 36). The results of our study were in agreement with the conclusions of the previously mentioned studies. Also, our research was in agreement with the results of other studies, where it had also been shown that Pd(II) complexes can induce cell cycle arrest in tumor cells (34, 37).

CONCLUSION

The results of our research demonstrated that Pd(II) complexes induced apoptosis via the mitochondrial pathway of apoptosis. All five investigated Pd(II) complexes, in particular C2, showed strong cytotoxicity against human prostate cancer cells in vitro. Thus, it is crucial to further investigate the cytotoxicity of these Pd(II) complexes in vivo. Complex C2 might be a good candidate for a new generation of anticancer drugs.

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RESPIRATORY REHABILITATION IMPROVES QUALITY OF LIFE AND FUNCTIONALITY OF COVID 19 PATIENTS

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ABSTRACT

Respiratory rehabilitation leads to reduction of symptoms, strengthens extremity musculature and improves emotional state and management of daily activities in patients with respiratory diseases. Aim of our study was to determine quality of life and functionality of COVID 19 patients before and after respiratory rehabilitation program. The study was conducted at the Clinical Center, Kragujevac, from June to July 2020. The study was a prospective clinical trial and included 62 patients with the acute-phase of COVID-19. Respiratory rehabilitation program started at hospital and continued at home for three months overall. Quality of life was measured by the EQ-5D-5L and patient's functionality by The FIM score. All five dimension of EQ-5D-5L were higher after respiratory rehabilitation program as well as EQ-5D index score and VAS score (0.8516±0.202 and 53.31±17.129 before rehabilitation, 0.9147±0.074 and 64.53±8.368 after rehabilitation). Respiratory exercise showed significantly improvement in FIM total score from 104.48±12.880 to 106.21±9.791, as well as in FIM motor and cognitive subscores. Respiratory rehabilitation program improves quality of life and functionality of COVID 19 patients.

Keywords: *COVID 19, EQ-5D-5L, FIM, respiratory rehabilitation.*

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (*SARS-CoV-2*) pneumonia significantly impairs the quality of life and functional status of patients. Even few months after onset of the SARS-CoV-2 infection, many patients are still affected with chronic, clinically relevant sequelae. The most frequently reported health issues are fatigue (53–87%), breathlessness (43–71%) and neuropsychological impairments (47%), with a high prevalence of psychological disorders such as increased levels of stress, anxiety and depression (1-4). The place and role of rehabilitation treatment is important not only in improving respiratory status but also in improving the quality of life and functionality after treatment of SARS-CoV-2 pneumonia(5).

The goal of respiratory rehabilitation in hospitalized Covid-19 patients is to improve dyspnea, relieve anxiety and depression, prevent complications, reduce morbidity, preserve functionality and improve quality of life. Early respiratory rehabilitation is not recommended for severe and critically ill patients if their condition remains unstable or progressively worsens, but for all other patients it is crucial. Individual approach to each patient is mandatory especially for elderly patients, obese patients and patients with multiple comorbidities. Evaluation and monitoring of the patient is carried out all the time during the rehabilitation (5).

It is well known that respiratory rehabilitation leads to reduction of symptoms, strengthens extremity musculature and improves emotional state and management of daily activities in patients with respiratory diseases (6). Aim of our study was to determine impact of Covid-19 infection on patient's quality of life and functionality and to investigate the efficacy of respiratory rehabilitation in improving them.

MATERIALS AND METHODS

Study design and participants

The research was conducted as prospective study at Department of physical medicine and rehabilitation, Clinical Center of Kragujevac, Serbia in June and July 2020. Participants included in study were patients in the post-acute phase of mild, moderate, severe or critical COVID-19 as defined by the World Health Organization (7) and treated in Clinical Center of Kragujevac. Inclusion criteria for participants were age 18 and above. Patient with heart failure (New York Heart Association classes III and IV), patient with cognitive and conscious impairment (Mini–Mental State Examination score below 21, Glasgow Coma Score below 13), patient with serious sight and hearing impairment and febrile patients were excluded from study. All patients give written consent and study wasapproved by the ethics committee of the Clinical Center of Kragujevac (no. 01/20/485 from 24/04/2020).

Intervention

Respiratory rehabilitation was conducted once a day and involves patient positioning, postural drainage, and breathing exercises. Oxygen saturation (SpO_2) and heart rate via a pulse oximeter were measured before and after treatment.

Breathing exercises were performed in the supine position with the legs bent at the knees, in a semi-sitting or sitting position according to patient's respiratory status. After positioning, a physical treatment with breathing exercises was applied - training in diaphragmatic breathing to establish breathing control, reducing the consumption of energy needed for breathing, and improving lung ventilation. The patient was instructed to inhale the air through the nose and exhale lightly through the mouth in the position of pronouncing the letter "O" so that the expiration would be prolonged, two to three times longer than the inspiration. This way of breathing leads to a control and reduction of dyspnea and reduction of respiratory rate. During the implementation of the exercise program, accessory muscles of the shoulders and neck are relaxed. Attention is paid to the expansion of the lower part of the chest and the mobilization of the upper extremities. These exercises help eliminate secretions from the airways and increase vital capacity.

After breathing exercises postural drainage was then performed with manual chest percussion and followed with another course of exercises. The rehabilitation program lasted between 20 and 45 minutes and was performed once a day. Respiratory rehabilitation was ended if patients complained on dyspnea (modified Borg dyspnea scale (MBS)> of 3), chest tightness, shortness of breath, blurred vision, palpitations, increased sweating and dizziness and other serious symptoms that physician estimate as contraindication for further exercises.

Respiratory rehabilitation was performed during patients' hospitalization and was continued at home by patients themselves.

Outcome and measures

Oxygen saturation and heart rate measurement

Oxygen saturation and heart rate were measured via a pulse oximeter before and 15-30 minutes after respiratory exercise program, by nurse.

Measurement of dyspnea

Measurement of dyspnea is performed using a modified ten-degree Borg scale for the assessment of dyspnea. It consists of ten verbal descriptors to which numerical values have been added, ranging from 0 to 10 (0 - no breathlessness, 10 - maximum breathlessness).

Quality of life assessment

Quality of life was measured using the EuroQol 5-Dimension 5-Level (EQ-5D-5L) (8). The EQ-5D-5L essentially consists of 2 pages: the EQ-5D descriptive system and the EQ visual analogue scale (EQ VAS). The descriptive system consists of five dimensions. The question regarding mobility asks about problems in walking. Regarding self-care, the question asks about problems in walking or dressing oneself. Regarding usual activities, the question asks about problems in performing one's usual activities such as study, work, familial duties, housework, or leisure activities. Regarding pain/discomfort, the question asks about having pain or discomfort in one's usual life. Finally, the question regarding anxiety/depression asks about any feelings of anxiety/depression. Each dimension has 5 levels: no problems (1), slight problems (2), moderate problems (3), severe problems (4) and extreme problems (5). The digits for the five dimensions can be combined into a 5-digit number that describes the patient's health state.EQ-5D-5L CrosswalkIndex Value Calculatorwas used to calculate index values for the EQ-5D-5Ldimension scores.

The EQ VAS records the patient's self-rated health on a vertical visual analogue scale from o which stands for "The worst health you can imagine" to 100 which means "The best health you can imagine".

In our study we performed first EQ-5D-5L measurement at patients' admission and second three months later using telephone.

Patient's functionality assessment

The Functional Independence Measure (FIM) is an 18-item instrument measuring a person's level of disability in terms of burden of care. FIM consist of 18 items, first 13 are forming motor subtotal score assessing independent performance in self-care, sphincter control, transfers, locomotion and last 5 are forming cognitive subtotal score assessing communication and social cognition. Each item is rated from 1 (requiring total assistance) to 7 (completely independent). After all items have been assessed, a total FIM score is calculated. Motor subtotal score can ranges between 13 and 91, while the cognitive component can range between 5 and 35, together forming total score from 18 to 126 (9).

In our study we performed first FIM score measurement at patients' admission and second three months later using telephone.

Statistics

Statistical analyses were performed using SPSS 26 (IBM, USA). Participant characteristics were reported as mean ± standard deviation (SD), or frequencies and percentages for categorical variables. The Chi-square goodness of fit test was used to determine the differences in the distribution of categorical variables. Continuous variables were compared between groups by the Mann-Whitney-U test. The Chi-square test of independence was used to examine the relationship between participant characteristics and domains. For comparing pre- to post-respiratory rehabilitation quality of life and patient functionality (EQ-5D-5L and FIM score), a two-tailed Wilcoxon rank-sum test was applied. A p-value less than 0.05 was considered to be a measure of statistical significance for all statistical tests used.

RESULTS

Our study included 62 patients, 38 males (61.3%) and 24 females (38.7%). Most of the patients were older than 60 years (n = 35; 56.45%) and most of the patients had hypertension, other comorbidities were less presented. The basic characteristics of the patients are given in Table 1.

The patient's a mean EQ-5D index score and VAS score before respiratory rehabilitation were 0.8516±0.202 and 53.31±17.129, respectively (Table 2).

The most frequently reported problem was anxiety/depression (80.6%) followed by pain/discomfort (79.1%). Self-care (21%) and usual activities were (21%) were the least frequently reported problems. Patients with previously diagnosed malignant diseases were more likely to report problem in mobility and self-care while patients with endocrinology diseases like Cushing disease, hipo- or hyperthyroidism diseases were more likely to report problem in self-care and usual activities. Sex, age, length of hospitalization and chronic diseases like hypertension, diabetes mellitus and hearth failure had no influence on five dimensions of EQ-5D before respiratory rehabilitation (Table 3).

After respiratory rehabilitation, both scores were higher, EQ-5D index score 0.9147±0.074 and VAS score 64.53±8.368 (Table 1).Still, most frequently reported problems were anxiety/depression (61.3%) and pain/discomfort (77.5%), but overall less percentage of patients reported those problems. Also patients reported fewer problems in mobility, self-care and usual activities (Table 4).

Comparing each dimension of EQ-5D, patients after respiratory rehabilitation showed significantly improvement in all five dimensions, mobility, self-care, usual activities, pain/discomfort and anxiety/depression (Table 2, Figure 1).

Respiratory rehabilitation showed improvement not only in quality of life but also in patient's functionality. FIM motor subtotal score showed significantly improvement from 70.90±13.365 before to 74.97±10.078 after respiratory rehabilitation. Similar findings were obtained in FIM cognitive subtotal score, respiratory rehabilitation improved score from 33.58±2.551 to 34.06±1.114. Finally we calculated FIMI total score before (104.48±12.880) and after (106.21±9.791) respiratory rehabilitation and we found that respiratory rehabilitation significantly improved overall patient's functionality, motor and cognitive (Table 5).

Figure 1. Five dimensions of EQ-5D before and after respiratory rehabilitation.

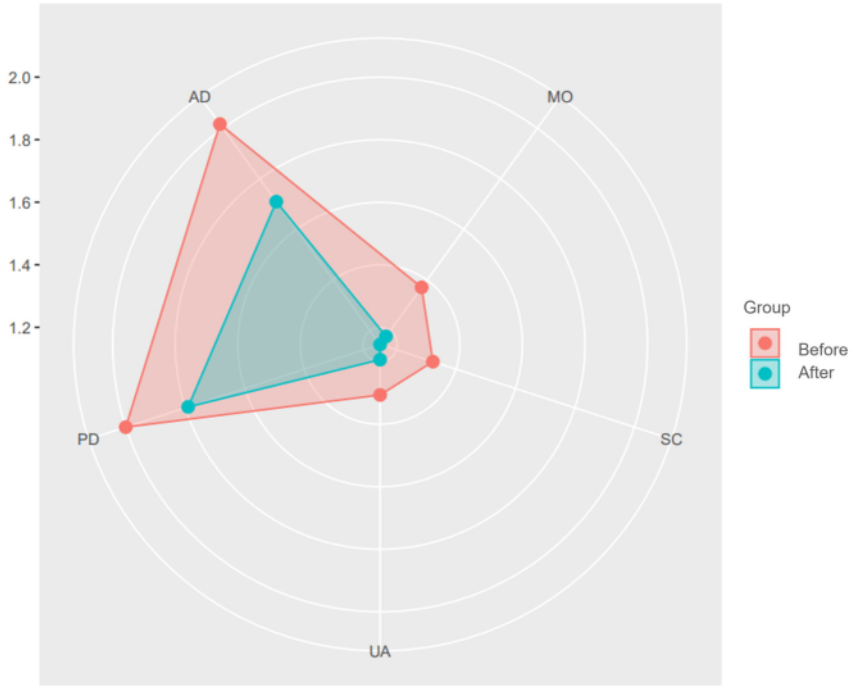

Table 1. Baseline demographic characteristics of the study population.

Characteristics		Results (%)	p-value
Gender	Male	38 (61.3 %)	p=0.075
	Female	24 (38.7%)	
Age	30-39	4 (6.45%)	p <0.001
	40-49	9 (14.52%)	
	50-59	13 (20.97%)	
	60+	35 (56.45%)	
Hypertension	Yes	31 (50%)	p=0.799
	No	31 (50%)	
Diabetes mellitus	Yes	16 (25.8%)	p <0.001
	No	46 (74.2%)	
COPD	Yes	6 (9.7%)	p <0.001
	No	56 (90.3%)	
Heart failure	Yes	5 (8.1%)	p <0.001
	No	57 (91.9%)	
Malignancy	Yes	10 (16.1%)	p <0.001
	No	52 (83.9%)	
Endocrine disorder	Yes	5 (8.1%)	p <0.001
	No	57 (91.9%)	

Table 2. Average value of all five dimensions of EQ-D before and after respiratory rehabilitation.

	Results (mean ± SD)	p-value
EQ-5D1 before	1.177±0.425	**0.010**
EQ-5D1 after	1.370±0.773	
EQ-5D2 before	1.145±0.437	**0.002**
EQ5-5D2 after	1.320±0.672	
EQ-5D3 before	1.193±0.437	**0.05**
EQ-5D3 after	1.310±0.781	
EQ-5D4 before	1.790±0.447	**0.009**
EQ-5D4 after	2.00±0.747	
EQ-5D5 before	1.709±0.662	**0.000**
EQ-5D5 after	2.020±0.689	
EQ-5D VAS	53.31±17.129	**0.000**
EQ-5D VAS	64.53±8.368	
EQ-5D Index before	0.8516±0.202	**0.000**
EQ-5DIndex after	0.9147±0.074	

Table 3. Percentage of reported any problem in 5 dimensions of EQ-5D before respiratory rehabilitation.

Characteristics		Mobility			Self-care			Usual Activities			Pain/discomfort			Anxiety/depression		
		No	Yes	p	No	Yes	p	No	Yes	p	No	Yes	p	No	Yes	p
Total		77.5	22.5		79	21		79	21		20.9	79.1		19.4	80.6	
Gender	Male	45.2	16.1	0.566	48.4	12.9	0.984	51.6	9.7	0.347	16.1	45.2	0.326	11.3	50.0	0.815
	Female	32.3	6.4		30.6	8.1		27.4	11.3		4.8	33.9		8.1	30.6	
Age	5.2	0.0	0.749	5.2 13.8 20.7 48.3	0.0	0.236	5.2 13.8 19.0 43.1	0.0	0.231	0.0 5.2 3.4 13.8	5.2	0.628	3.4 6.9 6.9 20.7	1.7	0.633	5.2 10.3 19.0 50.0
	10.3	3.4	5.2		0.0	1.7		0.0	1.7		8.6	8.6		6.9	13.8	
	19.0	1.7	1.7		0.0	0.0		1.7	1.7		17.2	17.2		13.8	17.2	
	50.0	10.3	15.5		12.1	20.7		17.2	19.0		46.6	48.3		39.7	44.8	
Lenght of hospitalization		13.94± 2.409	14.21± 1.805	0.868	14.00± 2.372	14.00± 1.958	0.761	14.04± 2.327	13.85± 2.154	0.584	13.85± 2.154	14.04± 2.327	0.667	13.25± 2.989	14.18± 2.68	0.494
Hypertension	Yes	35.5	14.5	0.224	38.7	11.3	0.755	37.1	12.9	0.349	9.7	40.3	0.755	8.1	41.9	0.520
	No	41.9	8.1		40.3	9.7		41.9	8.1		11.3	38.7		11.3	38.7	
Diabetes mellitus	Yes	17.7	8.1	0.538	21.0	4.8	0.800	19.4	6.5	0.646	8.1	17.7	0.241	4.8	21.0	0.943
	No	59.7	15.5		58.1	16.1		59.7	14.5		12.9	61.3		14.5	59.7	
COPD	Yes	6.5	3.2	0.610	6.5	3.2	0.597	0.0	9.7	0.328	1.6	8.1	0.861	6.5	1.6	0.884
	No	71.0	19.4		72.6	17.7		21.0	69.4		17.7	72.6		71.0	21.0	
Heart failure	Yes	6.5	1.6	0.886	8.1	0.0	0.352	8.1	0.0	0.352	3.2	4.8	0.280	1.6	6.5	0.970
	No	71.0	21.0		71.0	21.0		71.0	21.0		17.7	74.2		17.7	74.2	
Malignancy	Yes	8.1	8.1	**0.024**	8.1	8.1	**0.041**	9.7	6.5	0.196	3.2	12.9	0.934	3.2	12.9	0.955
	No	69.4	14.5		71.0	12.9		69.4	14.5		17.7	66.1		16.1	67.7	
Endocrine disorder	Yes	4.8	3.2	0.314	3.2	4.8	**0.050**	3.2	4.8	**0.050**	1.6	6.5	0.956	1.6	6.5	0.970
	No	72.6	19.4		75.8	16.1		75.8	16.1		19.4	72.6		17.7	74.2	

Table 4. Percentage of reported any problem in 5 dimensions of EQ-5D before respiratory rehabilitation.

Characteristics		Mobility			Self-care			Usual Activities			Pain/discomfort			Anxiety/depression		
		No	Yes	p	No	Yes	p	No	Yes	p	No	Yes	p	No	Yes	p
Total		83.9	16.1		88.7	11.3		82.2	17.8		22.5	77.5		38.7	61.3	
Gender	Male	50.0	11.3	0.727	53.2	8.1	0.696	53.2	8.1	0.311	17.7	43.6	0.212	24.2	37.1	0.876
	Female	33.9	4.8		35.5	3.2		29.0	9.7		4.8	33.9		14.5	24.2	
Age	30-39	5.2	0.0	0.426	5.2	0.0	0.050	5.2	0.0	0.341	0.0	5.2	0.630	0.0	5.2	0.443
	40-49	8.6	5.2		12.1	1.7		12.1	1.7		5.2	8.6		0.0	13.8	
	50-59	19.0	1.7		20.7	0.0		19.0	1.7		3.4	17.2		3.4	17.2	
	60+	44.8	15.5		39.7	20.7		41.4	19.0		12.1	48.3		15.5	44.8	
Lenght of hospitalization		14.00± 2.376	14.00± 1.764	0.789	13.98± 2.286	14.14± 2.340	0.965	14.00± 2.324	14.00± 2.145	0.782	13.93± 2.093	14.02± 2.347	0.739	13.83± 2.353	14.11± 2.252	0.922
Hypertension	Yes	40.3	9.7	0.731	43.5	6.5	0.688	40.3	9.7	0.739	11.3	28.7	0.999	14.5	35.5	0.118
	No	43.5	6.5		45.2	4.8		41.9	8.1		11.3	38.7		24.2	25.8	
Diabetes mellitus	Yes	19.4	6.5	0.266	24.2	1.6	0.666	21.0	4.8	0.903	8.1	17.7	0.336	8.1	17.7	0.561
	No	64.5	9.7		64.5	9.7		61.3	12.9		14.5	59.7		30.6	43.5	
COPD	Yes	8.1	1.6	0.970	8.1	1.6	0.528	8.1	1.6	0.942	0.0	9.7	0.322	3.2	6.5	0.776
	No	75.8	14.5		80.6	9.7		74.2	16.1		22.6	67.7		35.5	54.8	
Heart failure	Yes	6.5	1.6	0.811	8.1	0.0	0.405	8.1	0.0	0.575	3.2	4.8	0.314	1.6	6.5	0.640
	No	77.4	14.5		80.6	11.3		74.2	17.7		19.4	72.6		37.1	54.8	
Malignancy	Yes	9.7	6.5	**0.046**	9.7	6.5	**0.010**	9.7	6.5	0.067	3.2	12.9	0.831	4.8	11.3	0.727
	No	74.2	9.7		79.0	4.8		72.6	11.3		19.4	64.5		33.9	50.0	
Endocrine disorder	Yes	6.5	1.6	0.806	3.2	4.8	**0.008**	3.2	4.8	**0.035**	1.6	6.5	0.886	1.6	6.5	0.640
	No	77.4	14.5		85.5	6.5		79.0	12.9		21.0	71.0		37.1	54.8	

Table 5. FIMI subtotal scores and total score before and after respiratory rehabilitation.

	Mean ± SD	p-value
FIM motor subtotal score before	70.90±13.365	**0.000**
FIM motor subtotal score after	74.97±10.078	
FIM cognitive subtotal score before	33.58±2.551	**0.027**
FIM2 subtotal score after	34.06±1.114	
FIM total score before	104.48±12.880	**0.000**
FIM total score after	106.21±9.791	

DISCUSSION

Aim of present study was assessing EQ-5D score and FIM index before and after respiratory rehabilitation in patients with COVID 19 infection. The mean score for EQ-5D score and EQ-VAS scale before rehabilitation were 0.8516±0.202 and 53.31±17.129, respectively. When compared to EQ-5D values in patients with asthma (mean EQ-5D index: 0.77–0.88 and mean EQ-VAS: 57–67) (10,11), the current study demonstrate that quality of life is similarly effected in COVID-19 patients, even EQ-VAS values were better in asthma patients. The fact that majority of our patients had no pre-existing comorbidities, especially respiratory diseases, emphasizes the substantial persistent burden of COVID-19.

There are little data about long-term recovery from COVID 19 and quality of life of these patients. Previously studies showed that both non-hospitalized and hospitalized COVID 19 patients had lower quality of life (measured with EQ-5D) compared to general population three months after symptoms onset (12,13). These results clearly showed that COVID 19 patients had long-term problems that affected their everyday life. Our study demonstrated that respiratory rehabilitation could improve quality of life. EQ-5D index score and EQ-VAS score were higher after respiratory rehabilitation, 0.9147±0.074 and 64.53±8.368, respectively. These results are supported with previously studies that showed significantly improvement in respiratory parameters such as oxygen saturation, respiratory rate and need for oxygen therapy in COVID 19 patients after respiratory

rehabilitation (14,15). Comparing each dimension of EQ-5D, patients after respiratory rehabilitation showed significantly improvement in all five, mobility, self-care, usual activities, pain/discomfort and anxiety/depression. The most common problems our patients reported were anxiety and pain. Generally, mental health complications of anxiety, depression and poor sleep are often in acute phase of COVID 19 and in recovery phase due to biological and psychosocial factor (16). Another very often post-COVID 19 symptom are pain, including musculoskeletal pain, chest pain, headache, testicular pain (17-19). Our patients showed significant improvement regarding both, anxiety and pain after respiratory rehabilitation.

Patient's impairments in body functions and structure such as weakness, dyspnea, fatigue, chronic pain limit the ability to perform both, basic activities of daily living (ADL) and instrumental ADL. Basic ADL are related to personal care and mobility, whereas instrumental ADL are associated with the person's ability to interact with his environment (20). Functional Independence Measure score which was used in our research is commonly used scale to assess functional status regarding both ADL in critically ill patients (21).

Previously studies clearly showed that respiratory rehabilitation improved ADL in COVID 19 patients (22-24). Our research accordingly demonstrated that respiratory rehabilitation started in the hospital and continued at home significantly improved overall patient's functionality, motor and cognitive and improved patient's ability to perform ADL.

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Patients gave their written informed consent before the study enrollment.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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A STUDY OF ABSORPTION AND SELECTED MOLECULAR PHYSICOCHEMICAL PROPERTIES OF SOME ANTIPSYCHOTIC DRUGS

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ABSTRACT

Antipsychotic drugs are commonly prescribed for different mental disorders and can be classified into two main groups: the first which contain originally developed antipsychotics of the first generation or typical antipsychotics and the other group with newly developed antipsychotics or atypical antipsychotics of the second generation. In this study, eleven antipsychotic drugs (chlorpromazine, flupentixol, haloperidol, zuclopenthixol, aripiprazole, clozapine, olanzapine, quetiapine, risperidone, sertindole, ziprasidone) were investigated to evaluate significance of their molecular physicochemical properties (lipophilicity, aqueous solubility, polar surface area, molecular weight, volume value and acidity) for their bioavailability. Relationships between literature available intestinal absorption data of antipsychotic drugs and their lipophilicity descriptor with one additional molecular descriptor, investigated using multiple linear regression analysis provided high correlations for molecular descriptors, Mw, Vol, pKa, as additional independent variables. Values of correlation coefficients (R^2) were ranged from 0.951 (for Vol) above 0.944 (for Mw) to 0.923 (for pKa).

Keywords: *Antipsychotic drugs; absorption; lipophilicity; solubility; polar surface area.*

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INTRODUCTION

Psychotic disorders are today relatively frequent among global population. They can be divided into number of mental illnesses as psychoses, neuroses or many other. According to their mechanism of action antipsychotic drugs are generally antagonists of dopamine receptor. However, they can affect cholinergic, α adrenergic, histamine or serotonin receptors, as additional targets, which can influence their side effect.

From the beginning of their implementation until the 90's this drugs had been significantly improved and number of newly synthetized drugs were introduced into medical practice in aim to optimize their efficiency, safety and modes of application (1-5).

Concerning their development antipsychotic drugs can be divided into two main groups. There are originally developed antipsychotics which belong to the first group of drugs, known as typical antipsychotics or antipsychotics of the first generation (chlorpromazine, flupentixol, haloperidol, zuclopenthixol and others), while the other group represents newly developed antipsychotics, known as atypical, namely antipsychotics of the second generation (aripiprazole, clozapine, olanzapine, quetiapine, risperidone, sertindole, ziprasidone and others) (1-5).

It is generally known that medical success and therapeutic efficiency of drugs significantly depend on their absorption, distribution, metabolism and route of elimination namely their ADME data (6-8). On the other hand, drugs ADME properties are critically influenced by physicochemical properties of drugs molecules: lipophilicity, solubility, molecular weight, volume and acidity (8-12).

In our prior researches, influence of several molecular physicochemical properties on absorption (13-15), plasma protein binding (16,17) as well as route of elimination (18-20) were investigated for number of antihypertensive drugs and significant dependence was established. Following in our recently published paper we studied relationship between antipsychotics molecular properties and their plasma protein binding degree (21).

The the aim of our study was to estimate molecular physicochemical descriptors of several antipsychotic drugs and to compare their values with each other and published literature data about their absorption.

MATERIALS AND METHODS

In this investigation eleven antipsychotic drugs were selected, four which belong to the typical or antipsychotics of the first generation and seven atypical or antipsychotics of the second generation. The eleven selected antipsychotics investigated in presented research were: **1.** chlorpromazine, **2.** flupentixol, **3.** haloperidol, **4**. zuclopenthixol, and seven atypical: **5.** aripiprazole **6.** clozapine, **7.** olanzapine, **8.** quetiapine, **9.** risperidone, **10.** sertindole and **11.** ziprasidone.

For calculation of antipsychotics' molecular physicochemical descriptors, several software packages were used: Virtual Computational Chemistry Laboratory (22), Molinspiration Depiction Software (23), as well as DrugBank (24) a database of published data on the pharmacological drugs properties and Chemdraw ultra 12.0.

With application of software package Molinspiration Depiction Software (www.molinspiration.com) three molecular physicochemical descriptors were calculated: electronic descriptor - polar surface area (*PSA*); constitutional parameter - molecular weight (*Mw*) and geometric descriptor - volume value (*Vo*l) (23).

Using software package, Virtual Computational Chemistry Laboratory (www.vcclab.org) antipsychotics' lipophilicity descriptors, seven different log*P* values, as well as their aqueous solubility data (log*S*) were calculated (22). The additional lipophilicity parameter was calculated with application of Chemdraw ultra 12.0 software package while experimental lipophilicity parameters (log*Pexp*) of investigated antipsychotics were obtained using DrugBank database (24).

The DrugBank database was also used to obtain antipsychotics' acidity descriptors, pK_a values. Moreover, data about values of intestinal absorption of investigated antipsychotics were obtained using DrugBank database (24).

RESULTS

The calculated lipophilicity descriptors (different log*P* values) and aqueous solubility data (log*S* values) are presented in Table 1, intercorrelations between all colected log*P* values of investigated antipsychotic drugs are presented at Table 2, while antipsychotics data of polar surface area (*PSA*), molecular weight (*Mw*), volume value (*Vol*) and antipsychotics' acidity descriptors (pK_a values) as well as their bioavailability, are presented in Table 3.

All statistical analysis were performed by application the Microsoft Excel 2003 and Origin 7.0 PRO (Origin Lab Corporation, USA).

Table 1. The lipophilicity (logP) and solubility (logS) values of investigated antipsychotic drugs

No.	Compound	logP exp	ClogP	AlogPs	AClogP	milogP	AlogP	MlogP	XlogP2	XlogP3	log*S*
1	Chloropromazine	5.41	5.80	5.18	5.03	5.03	4.74	3.77	4.92	5.19	-4.84
2	Flupentixol	4.51	4.34	4.56	4.45	4.91	4.82	3.89	4.42	4.51	-4.50
3	Haloperidol	4.30	3.85	3.70	4.63	4.30	3.89	4.01	3.98	3.23	-4.81
4	Zuclopenthixol	nd	4.13	4.46	4.30	4.69	4.54	3.58	4.12	4.31	-4.55
5	Aripiprazole	4.50	4.63	5.21	4.58	5.08	5.00	3.61	4.49	4.64	-4.95
6	Clozapine	3.23	4.10	3.67	3.21	4.14	3.95	2.96	3.74	3.08	-3.26
7	Olanzapine	2.00	3.40	3.61	2.98	3.47	3.21	2.31	2.32	2.86	-3.28
8	Quetriapine	2.80	3.37	2.93	2.80	3.49	3.18	2.36	2.83	2.14	-3.22
9	Risperidone	2.50	2.71	2.41	3.37	2.96	3.32	3.61	3.07	2.72	-3.89
10	Sertindole	nd*	5.07	4.29	4.52	3.84	4.68	3.77	4.10	4.07	-5.78
11	Ziprasidone	3.80	3.58	4.64	2.45	4.05	4.26	3.44	3.77	4.02	-4.32

* no data

Table 2. Intercorrelations between different log*P* values of investigated antipsychotic drugs

	logP exp	ClogP	AlogPs	AClogP	milogP	AlogP	MlogP	XlogP2	XlogP3
logP exp	1								
ClogP	0.8548	1							
AlogPs	0.8062	0.8297	1						
AClogP	0.7897	0.7331	0.5279	1					
milogP	0.9099	0.8913	0.9080	0.7504	1				
AlogP	0.8861	0.7914	0.9067	0.6666	0.9418	1			
MlogP	0.7545	0.4027	0.4322	0.7107	0.5626	0.6745	1		
XlogP2	0.9704	0.8353	0.7882	0.7741	0.9065	0.9296	0.7891	1	
XlogP3	0.8693	0.8369	0.9275	0.6699	0.8812	0.9426	0.6495	0.8822	1

Table 3. Intestinal absorption, calculated values of polar surface area, volume, molecular weight, acidity descriptors (pK_a values for pH = 7.4) for investigated antipsychotic drugs

No.	Compound	% ABS	PSA	Vol	Mw	pKa
1	Chloropromazine	10-80*	8.17	285	319	9.20
2	Flupentixol	47	26.70	379	435	8.51
3	Haloperidol	65	40.54	337	376	8.05
4	Zuclopenthixol	49	26.70	361	401	8.43
5	Aripiprazole	87	44.81	395	448	7.46
6	Clozapine	65	35.16	292	327	7.50
7	Olanzapine	87	35.16	286	312	7.24
8	Quetriapine	100	48.83	352	384	7.06
9	Risperidone	70	64.17	374	410	8.76
10	Sertindole	75	40.41	390	441	8.59
11	Ziprasidone	60	48.47	352	413	7.09

* absorption values have large individual variation

DISCUSION

Drugs Lipophilicity, Solubility and acidity

In this study, eleven antipsychotic drugs, four which belong to the typical or antipsychotics of the first generation (chlorpromazine, flupentixol, haloperidol, zuclopenthixol) and seven atypical or antipsychotics of the second generation (aripiprazole, clozapine, olanzapine, quetiapine, risperidone, sertindole, ziprasidone) were investigated to evaluate influence of their molecular physicochemical properties on their bioavailability.

The molecular structure and physicochemical properties of drugs molecules, at the first place its lipophilicity, play important role in development of new antipsychotic drugs. The calculation of lipophilicity descriptors, seven different log*P* values (Alog*Ps,* AClog*P,* milog*P*, Alog*P,* Mlog*P,* XLOG*P*2*,* XLOG*P*3) were performed using software package Virtual Computational Chemistry Laboratory. The another software package, Chemdraw ultra 12.0 was applied for calculation of Clog*P* lipophilicity parameter, while DrugBank database was used to obtain experimental lipophilicity parameters, logPexp values.

The relations between all obtained log*P* values were studied (27) and calculated correlation coefficients showed that good agreements were obtained between majorities of collected log*P* values, but the best can be observed for Xlog*P2* data. Also, it can be seen that although all calculated log*P* values correlate well with experimental log*P* data collected for investigated antipsychotic drugs and the best correlation for experimental log*P* was obtained with calculated Xlog*P2* (R^2 = 0.971). All collected log*P* values demonstrate similar trend. The typical, antipsychotic drugs from the first generation are more lipophilic then atypical or antipsychotics from second generation. According calculated log*P* values, the most lipophilic antipsychotic drug is typical antipsychotic chloropromazine, while the less lipophilic ones are atypical antipsychotics olanzapine and quetriapine.

Furthermore, another one molecular property, water solubility has important influence on drug's absorption as well as transport from site of administration to the blood. The low drug's absorption can be result of its insufficient water solubility (25,26). As can be seen, according calculated solubility log*S* values of antipsychotic drugs which are presented in Table 1, the lowest solubility value show atypical antipsychotic sertindole. However, results generally demonstrate that investigated atypical antipsychotics exhibit higher water solubility values than typical ones. Among typical, antipsychotics from the first generation, chloropromazine shows the lowest solubility which correspond to its highest lipophilicity, while the highest water solubility show atypical antipsychotic quetriapine what is in accordance with its low lipophilicity.

The drug's partition are significantly influenced by its acidity and dissociation (28). The calculated p*Ka* values in water at temperature of 25.0°C for all investigated antipsychotics showed general trend. The calculated acidity, p*Ka* values of antipsychotic drugs, show that the lowest value of p*Ka* (7.06) exibit quetriapine and for majority of investigated atypical antipsychotics p*Ka* values are lower than 7.5. The exceptions represent risperidone and sertindole (with p*Ka* values higher than 8.0). However, typical, antipsychotics from the first generation exhibit higher p*Ka* which are ranged from 8.1 for haloperidol to 9.2 for chloropromazine. Among antipsychotics from the first generation (typical) chloropromazine already showed the lowest solubility and highest lipophilicity values.

Polar surface area, molecular weight, volume

Beside lipophilicity, solubility and acidity for investigated antipsychotic drugs, further molecular descriptors, polar surface area (*PSA*), molecular weight (*Mw*) as well as molecular volume (*Vol*) were calculated. All these molecular properties together with lipophilicity, solubility and acidity have significant influence and correlates good with the human intestinal absorption (25,26). According investigation of number of drugs which belong to different groups it was supposed that very high values of *PSA, Mw or Vol* can lead to poor drug absorption (25,26).

Among drugs investigated in present research the lowest value of *PSA* exhibit typical antipsychotics chloropromazine (8.17) while flupentixol and zuclopenthixol show four times higher values of *PSA*. However majority of atypical antipsychotics have showen PSA values in the range 35.16 (for clozapine and olanzapine) to 64.17 (risperidone).

According presented results for all investigated antipsychotic drugs, molecular weight values are in the range 312 (olanzapine) to 448 (aripiprazole) while volume values are ranged from 285 (chloropromazine) to 395 (aripiprazole). For both parameters, molecular weight as well as volume value, the lowest values for typical antipsychotics belong to chloropromazine while highest values have been calculated for atypical antipsychotic aripiprazole.

Oral absorption

The main route of drugs administration generally is oral application. According Lipinski's "the rule of 5" low absorption or permeation can be predicted for drugs with molecular weight larger than 500 and the calculated log*P* higher than 5, but also for those with more than 5 hydrogen-bond donors, 10 hydrogen-bond acceptors (12). Also insufficient water solubility or very high values of *PSA* and *Vol* values can lead to poor drug absorption (25,26). Molecular physicochemical properties of drugs are important factors which can modulate their intestinal absorption.

The intestinal absorption values for all investigated antipsychotic drugs according available literature are in the range 10 to 100%. The majority of investigated antipsychotic drugs exhibit values of intestinal absorption in the range 50-80%. However, typical, antipsychotic drugs from the first generation, show lower values of intestinal absorption than atypical, antipsychotic drugs from the second generation.

Among investigated antipsychotic drugs of second generation, aripiprazole, olanzapine, quetriapine, are those with highest values of intestinal absorption. Their molecular weights are lower than 500 and in accordance with Lipinski's "the rule of 5". Among atypical antipsychotics aripiprazole is molecule with highest lipophilicity while the less lipophilic ones are olanzapine and quetriapine. Their polar surface area values are ranged from 35 to 49 and volume values from 286 to 394. However, the molecular parameter that distinguishes this three antipsychotic drugs of second generation, from other especially typical antipsychotics, is their acidity, namely their p*Ka* values which are in the range 7.46 – 7.06 while physiological pH is 7.4.

On the other hand chloropromazine value of intestinal absorption can differ 10-80% (absorption values have large individual variation). Chloropromazine belongs to typical or antipsychotic drugs of the first generation and already was pointed as a drug with highest p*Ka* value, low solubility and highest lipophilicity. Its value of Xlog*P2* was 4.92, what is very close to value of 5 and it is known that Lipinski's "the rule of 5" predict drugs low absorption for log*P* values higher than 5.

Since values of intestinal absorption for investigated antipsychotic drugs are highest for antipsychotics from second generation, especially for aripiprazole, olanzapine, quetriapine which have shown high solubility, moderate lipophilicity and molecular weight as well as p*Ka* values around 7.4, this can indicate that molecular physicochemical properties, lipophilicity, solubility, molecular weight and acidity, p*Ka*, but also polar surface area and volume are very important factors which are decisive for drug absorption.

In final stage of study, the relationships between all calculated molecular descriptors (lipophilicity, solubility, polar surface area, molecular weight, volume and acidity) of selected drugs and data about their intestinal absorption were examined using simple linear regression. The correlations between intestinal absorption data and antipsychotic drugs calculated molecular descriptors, *PSA, Mw, Vol, logS, pKa* were investigated providing not very good correlations with correlation coefficients (R^2) lower than 0.38.

The slightly better correlations were obtained between antipsychotic drugs intestinal absorption data and several calculated log*P* values. The best correlations were obtained for values of Mlog*P* (R^2 = 0.445) and Xlog*P2* (R^2 = 0.398). Since Xlog*P2* values have already shown the best correlation (R^2 = 0.971) with experimental logP data collected for investigated antipsychotic this lipophilicity descriptor (Xlog*P2*) was chosen for further study. Following, the correlation between antipsychotic drugs intestinal absorption, lipophilicity descriptor Xlog*P2* and one additional molecular descriptor were investigated using multiple linear regression analysis (MLR). As potential additional descriptors *Mw, Vol* and p*Ka* were chosen, since there were found relatively good correlations (R^2 > 0.30) between Xlog*P2* and values of log*S* and *PSA*.

Relationships investigated using multiple linear regression analysis provided considerably higher correlations for all used molecular descriptors, *Mw, Vol*, p*Ka*, as additional independent variables. Values of correlation coefficients (R^2) were ranged from 0.951 (for *Vol*) above 0.944 (for *Mw*) to 0.923 (for p*Ka*). All established correlations with obtained correlation coefficient R^2 higher than 0.80 can be considered as very good, as proposed by Asuero et al. (29), especially due to the limited number of compounds, eleven investigated antipsychotic drugs. The all results obtained using MLR analysis applying two different descriptors (*Mw, Vol*, p*Ka*) as independent variables are presented in Table 4 and at Figure 1.

The selection criteria for drug – like properties of investigated antipsychotic drugs are reported in Table 5 make it obvious that in the aim to obtain a potent antipsychotic drug, appropriate balance between physicochemical and pharmacokinetic properties should be established.

Table 4 . The antipsychotic drugs intestinal absorption data collected from literature (*) and predicted using (A) XlogP and Vol data; (B) XlogP2 and Mw; (C) XlogP and pKa values

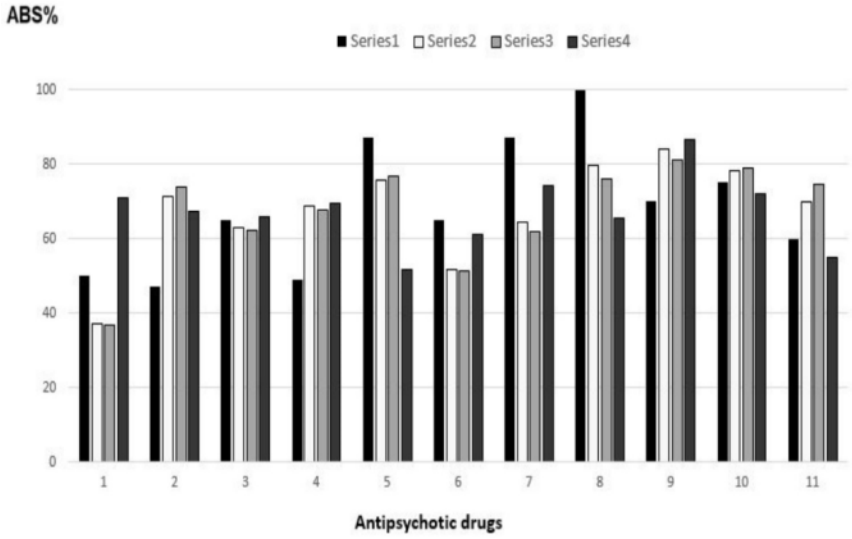
No.	Compound	ABS%(*)	ABS% (A)	ABS% (B)	ABS% (C)
1	Chloropromazine	50*	37	37	71
2	Flupentixol	47	71	74	67
3	Haloperidol	65	63	62	66
4	Zuclopenthixol	49	69	68	70
5	Aripiprazole	87	76	77	52
6	Clozapine	65	52	51	61
7	Olanzapine	87	65	62	74
8	Quetriapine	100	80	76	66
9	Risperidone	70	84	81	87
10	Sertindole	75	78	79	72
11	Ziprasidone	60	70	75	55

*average value used for calculation

Table 5. Drug - like properties of investigated antipsychotic drugs

Lipophilicity descriptor (Xlog*P2*)	2.32 – 4.92
Aqueous solubility (log*S*)	(-5.78) – (-3.22)
Acidity descriptor (pKa)	7.06 – 9.20
Polar surface area (PSA, Å^2)	8.17 – 64.17
Molecular weight (Mw)	312 – 448
Volume value	285 – 395
Percent of oral absorption (%ABS)	10 – 100

Figure 1. The relationship between antipsychotic drugs intestinal absorption data collected from literature (seria 1) and predicted using (seria 2) XlogP2 and Vol; (seria 3) XlogP and Mw data (seria 4) XlogP and pKa values. No denote investigated antipsychotic drugs



CONCLUSION

The investigation of relationship between literature available intestinal absorption data of antipsychotic drugs, and lipophilicity descriptor (Xlog*P2*) with one additional molecular descriptor (*Mw, Vol* or p*Ka*) provided high correlations (with $R^2 > 0.9$) and confirmed that application of in silico achieved molecular descriptors can be valuable technique in drug research and development of new drugs candidates.

CONFLICT OF INTEREST

The author declare no conflict of interest.

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EVALUATION OF MORPHOMETRIC PARAMETERS IN PCOS RATS TREATED WITH STANDARDIZED *ARONIA MELANOCARPA L.* EXTRACT AND/OR METFORMIN

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ABSTRACT

Polycystic ovary syndrome (PCOS) represents endocrine disorder which impacts women in the reproductive age. Due to the side effects of medications and the subsequent discontinuation of therapy, the influence of alternative medicine is increasing. The aim of this study was to investigate morphometric parameters and ovarian and adipose tissue histological structure in rats with polycystic ovary syndrome treated by standardized Aronia melanocarpa extract (SEA) and/or metformin. 24 animals with induced PCOS were divided into 4 groups: PCOS group, PCOS+MET group (treated with metformin), PCOS+SEA group (treated with aronia melanocarpa extract), and PCOS+MET+SEA (treated with metformin and aronia melanocarpa extract). Final body weight and body weight gain were significantly lower after all three type of treatments. Ovary weight was reduces in all three treated groups, while relative ovary weight was significantly lower only in SEA treated rats. However, both MET treated groups expressed lower adipocyte area, while adipocyte diameter was lowered only after combined treatment. Lower number of cysts and greater number of corpora lutea were registered in all treated groups. Our study highlights the significant impact of these interventions on morphometric parameters, indicating their potential to address obesity, a prevalent comorbidity in PCOS. Notably, the reduction in adipocyte size and the modulation of adipose tissue morphology suggest a potential avenue for ameliorating metabolic dysregulations associated with PCOS. Future research endeavors should aim to comprehensively address the multifaceted nature of this syndrome, with a view towards developing integrated therapeutic approaches that offer renewed hope for individuals grappling with PCOS-related challenges.

Keywords: *Polycystic ovary syndrome, metformin, aronia melanocarpa, dehydroepiandrosterone, antioxidants, animal model, rats.*

INTRODUCTION

Polycystic ovary syndrome (PCOS) represents endocrine disorder which impacts women in the reproductive age (1). The current PCOS incidence is about 7-10% of the female population (2). Beside polycystic ovaries (PCO), this condition is mostly characterized by the presence of hyperandrogenism and ovulatory dysfunction. To diagnose PCOS it is necessary to be present at least two of these disorders. PCOS can cause cardiovascular diseases, as well as metabolic disorders such as obesity, hyperinsulinemia, diabetes mellitus type 2 (3). PCOS is stated as one of the most important causes of infertility in women (4).

The exact etiology of PCOS is still unknown (3). Many earlier studies suggested that genetics and environmental factors ad a major impact in PCOS development (5-8). It is believed that PCOS is passed down between fertile carrier males and subfertile females (9). That claim can explain the high familial incidence of diabetes mellitus type 2 and hyperandrogenism in first-degree relatives of women with PCOS. In addition, it is found a single-nucleotid polymorphism rs 13429458 associated with a greater risk of PCOS development (10). Unhealthy habits such as smoking, absence of exercise or obesity are also risk factors for PCOS (11). The highest incidence of PCOS is reported among women from Central and North America, which indicates that ethnicity could have an influence on PCOS (12).

Anovulation is still the most common clinical sing of PCOS. More than 90% patients with PCOS has some type of anovulation (13). Usually it is represented as oligomennorrhea with a different number of periods in one year, usually not more than eight. Hyperandrogenism is manifested with increased blood levels of androgens, which is clinically seen as hirsutism, acne, alopecia (14). Polycystic ovaries are the third criterion when diagnosing PCOS. It is defined as a presence of more than 12 follicles in each ovary, dimensions 2-9 mm and/or enlarged ovary volume over 10 ml (15). It is very important to know that not every polycystic ovaries are necessary associated with PCOS. Only those correlated with anovulation and/or hyperandrogenism can be diagnosed as PCOS.

Currently the usage of combined oral contraceptive pills is the first line in PCOS treatment. Those medications could regulate a menstrual cycle and decrease a production of androgens (16). Insulin-sensitizing agents and metformin (MET) therapy are also included in PCOS treatment (17). In addition, physical activity, correction of diet will help to eliminate cardiovascular and other disorders associated with PCOS.

There is a strong correlation between PCOS and obesity, as many women with this condition are reported to be overweight or obese. It is believed that obesity play a central role in the development of PCOS. Insulin resistance and hyperandrogenism, some of metabolic and hormonal disorder that are present, in women predisposed to PCOS, can lead to weight gain and eventually obesity. This indicates that obesity may be an important predictor of PCOS, and obesity, in turn, can worsen PCOS symptoms, such as further metabolic disorder and reproductive abnormalities (18).

In women with PCOS chronic excess of ovarian and/or adrenal androgens leads to an increase in the amount of abdominal adipose tissue and androgenic obesity (19). Abdominal adipose tissue promotes the release of inflammatory mediators and consequently excess androgens through the direct response of the ovaries and adrenal glands to inflammatory mediators or indirectly through the development of insulin resistance and compensatory hyperinsulinemia (20), which is a vicious cycle of PCOS and its metabolic comorbidities.

Due to the appearance of side effects of medications and the subsequent discontinuation of therapy, the influence of alternative medicine is increasing. Recent studies investigated the possible effect of some novel compounds, such as Aronia melanocarpa treatment of various disorders (21, 22). A fresh fruits of Aronia melanocapra are not suitable to be consumed due to their bitter taste. These berries are usually used as juice, jam, fruit tea. It is known that they are rich in phenolic substances (23). Among them are procyanidins, phenolic acids, anthocyanins. These compounds show potent antioxidant and immunomodulatory effects, so they can be used as adjuvant therapy in various diseases (24) (25). Knowing that oxidative stress and subclinical inflammation have their role in PCOS pathogenesis gives space to explore an effect of Aronia melanocapra in PCOS treatment (6).

The aim of this study was to investigate morphometric parameters and ovarian and adipose tissue histological structure in rats with polycystic ovary syndrome treated by standardized Aronia melanocarpa extract (SEA) and/or MET.

MATERIAL AND METHODS

Ethics statement

Experiments were conducted in accordance with the Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac (Number of Ethical approval 01-17057, date 27.17.2019.). All experimental procedures were in accordance with the prescribed acts (EU Directive for the Protection of the Vertebrate Animals used for Experimental and other Scientific Purposes 86/609/EES) and principles of the Good Laboratory Practice.

Animals

The animals were obtained from the Military Medical Academy, Belgrade, Serbia. During the research, the animals were kept in the vivarium of the Faculty of Medical Sciences, University of Kragujevac. Female Wistar albino rats were used in this research. The animals were 6 weeks, had an average body weight (BW) of 150-170 g and. They were kept under controlled conditions (temperature 23±1 ^{0}C, 12:12h light/dark cycle – lights on 08:00h, air humidity of 55±5%)

with unlimited access to food and water (*ab libitum*). Rats of this age belong to the category of post-pubertal animals.

The study included 36 animals divided into two groups: control group (n=6) and PCOS group (n=30). After applied therapy for an induction of PCOS, 6 animals from PCOS group and 6 animals from control group were sacrificed for the purpose of verification of the changes caused by PCOS. After that, the rest of 24 animals with PCOS were further divided into 4 groups according to applied therapy:

PCOS group (n=6, PCOS animals treated only with distilled water),
PCOS + MET group (n=6, PCOS animals treated with metformin),
PCOS + SEA group (n=6, PCOS animals treated with aronia melanocarpa extract),
PCOS + MET + SEA (n=6, PCOS animals treated with metformin and aronia melanocarpa extract).

PCOS induction and treatment

PCOS was induced by daily subcutaneous injections of dehydroepiandrosterone (DHEA, Millipore, Darmstadt, Germany; 60 mg/kg of body weight) dissolved in 0.2 mL of sesame oil (Sigma-Aldrich, St. Louis, MO, USA) during 5 weeks. In order to confirm PCOS, animals from the control group, as well as 6 animals from the PCOS group, were sacrificed by decapitation on the guillotine, after applying appropriate anesthesia with an intraperitoneal injection of a mixture of ketamine and xylazine (50 mg/kg ketamine and 100 mg/kg xylazine). Biochemical and histological analyzes were carried out in order to confirm the induction of PCOS in rats (hyperandrogenemia, ovary cysts, absence of corpora lutea). Cytological examination of the vaginal smears was performed during the last 12 days of PCOS treatment, in order to identify the phase of the estrous cycle.

MET (Sigma, Aldrich) was dissolved in distilled water and applied at a dose of 500 mg/kg of body weight (BW) daily (26). SEA (Pharmanova, Belgrade, Serbia) was administered at a dose of 0.45 mL/kg of BW daily (27). The extraction of SAE was performed by EU-Chem Company (Belgrade, Serbia). Based on quantitative and qualitative analyses, the following individual compounds were found in SAE and expressed in mg/mL of extract: 2.68 cyanidin 3-galactoside (80.40 mg/ mL), 0.16 cyanidin 3-glucoside (4.92 mg/mL), 0.66 cyanidin 3-arabinoside (19.71 mg/mL), 0.14 cyanidin 3-xyloside (4.26 mg/mL), 0.12 rutin (3.55 mg/mL), 0.27 hyperoside (8.12 mg/mL) and 0.15 isoquercetin (4.36 mg/mL) (27, 28]. Total amount of polyphenols is 410 mg/30 mL. A study protocol lasted for 28 days. At the end all animals were decapitated using the guillotine, after receiving appropriate anesthesia.

Assessment of body weight parameters

Twice a week during, the body weight of the animals was measured. Also, the BW was measured just before sacrifice. Body mass gain was calculated as a percentage of increase compared to initial body weight values. The body length was measured at the end of the experimental protocol, using a flexible centimeter from the tip of the nose to the anal (nasoanal length), while the animals were under anesthesia, just before sacrifice. Body mass index (BMI) calculated based on the data collected by measuring body weight and body length: BMI = body weight / body length^2. Body fat index (Lee index) was calculated using 3 square root body weight (g) / nasoanal length (cm) x 1000.

Assessment of estrus cycle

The stages of the estrous cycle were assessed by cytological examination of vaginal smears. Vaginal irrigation was performed using a glass pipette which was filled with a small amount of saline. The wash was mounted on a glass slide, air-dried and counterstained with hematoxylin. After drying, the analysis was carried out using a light microscope. The phases of the estrous cycle are identified according to the predominance of specific cells on the preparation: proestrus — dominantly present round cells with a nucleus, estrus — dominantly present horny squamous cells, metaestrus - approximately the same representation of horned squamous cells and leukocytes, diestrus - the presence of epithelial cells with a nucleus and the dominance of leukocytes (28). Estrous cycle assessment was performed during 12 consecutive days of the PCOS induction protocol and then the PCOS treatment protocol. Also, at the beginning of the study protocol, in order to select animals that have an orderly estrous cycle and include them in the research, before any treatments, the phase of the estrous cycle was checked.

Histological analysis of ovaries and subcutaneous fat tissue

The weight of the left ovary was measured after isolation of the organ on an analytical scale, while relative ovarian weight was calculated as the quotient of ovarian weight and body weight. The left ovary and subcutaneous fat tissue were isolated, weighed and fixed in 4% formaldehyde at room temperature. After the fixation, the tissue samples was dehydrated through a series of alcohols of increasing concentration (50, 70, 96 and 100%), illuminated in xylene and molded in paraplast. Transverse serial sections 5 micrometers thick were cut on a rotary microtome. After drying, the tissue sections were deparaffinized in xylene, rehydrated in decreasing concentrations of alcohol (100, 96, 70, 50%) and washed in water. Then they were stained by using hematoxylin and 2% eosin solution. After staining, sections were mounted with DPX and coated by cover glass. Then morphometric analysis was performed in order to quantify number of cystic follicles, as well as the number of corpora lutea using a light microscope (Olympus BX51). Adipocyte area and diameter were assessed by Image J Adiposoft Software.

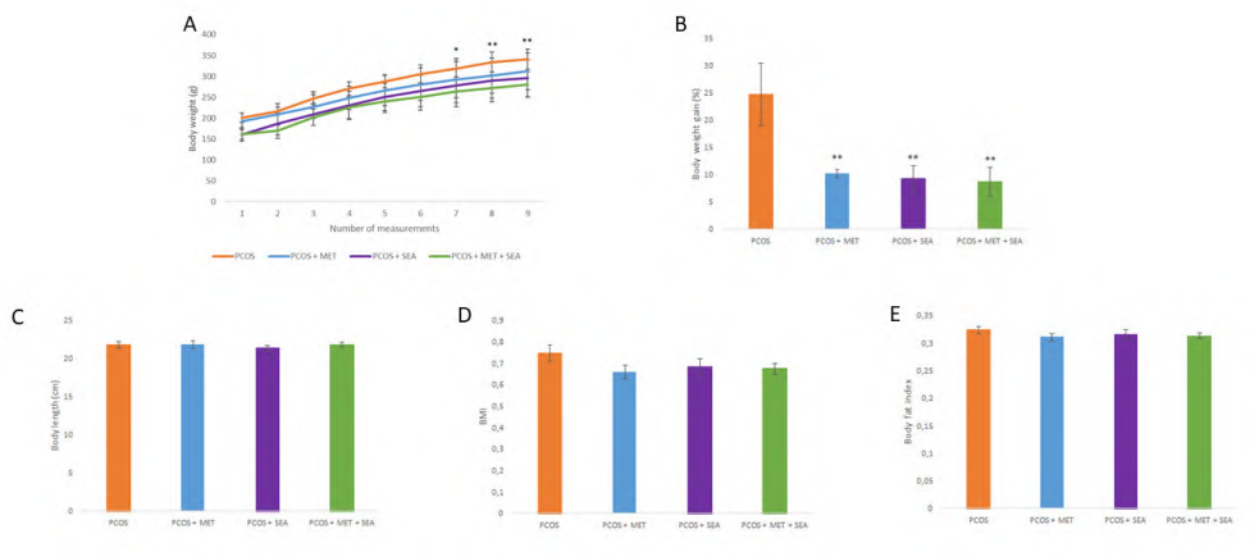
Statistical analysis

The data were analyzed with SPSS statistical software (version 22). Depending of the normality all date were analyzed using ANOVA or Kruskal-Wallis test. A *p* value of less than 0.05 was considered as statistically significant, and *p* value of less than 0.01 was considered to be highly statistically significant.

RESULTS

The body weight of the animals was measured twice a week, during four weeks of treatment, and results are presented in Figure 1A. There was no statistically significant difference in body weight within each individual group during the protocol. The comparison between the groups showed a statistically significantly higher body weight of the PCOS group compared to the PCOS + MET, PCOS + SEA and PCOS + MET + SEA groups ($p<0.05$ in the 7th measurement, as well as $p<0.001$ in the 8th measurement). At the end of the experiment, the final body weight was significantly higher in the PCOS group compared to the other groups (9th measurement, $p<0.01$). Body weight gain in animals with PCOS was statistically significantly lower after the treatment with metformin ($p<0.01$), and Aronia melanocarpa extract ($p<0.01$), as well as after their combined treatment ($p<0.01$), compared to the animals treated only with distilled water (Figure 1B). No statistically significant difference was observed in body length, body mass index and body fat index among the groups ($p>0.05$) (Figures 1C, 1D, 1E).

Figure 1. The influence of usage metformin and Aronia melanocarpa extract, as well as their combined treatment on body weight parameters.



A – body weight; B –body weight gain; C – body length; D – body mass index; E – body fat. * Statistical significance at the level of $p < 0.05$ compared to PCOS group, ** Statistical significance at the level of $p < 0.01$ compared to PCOS group.

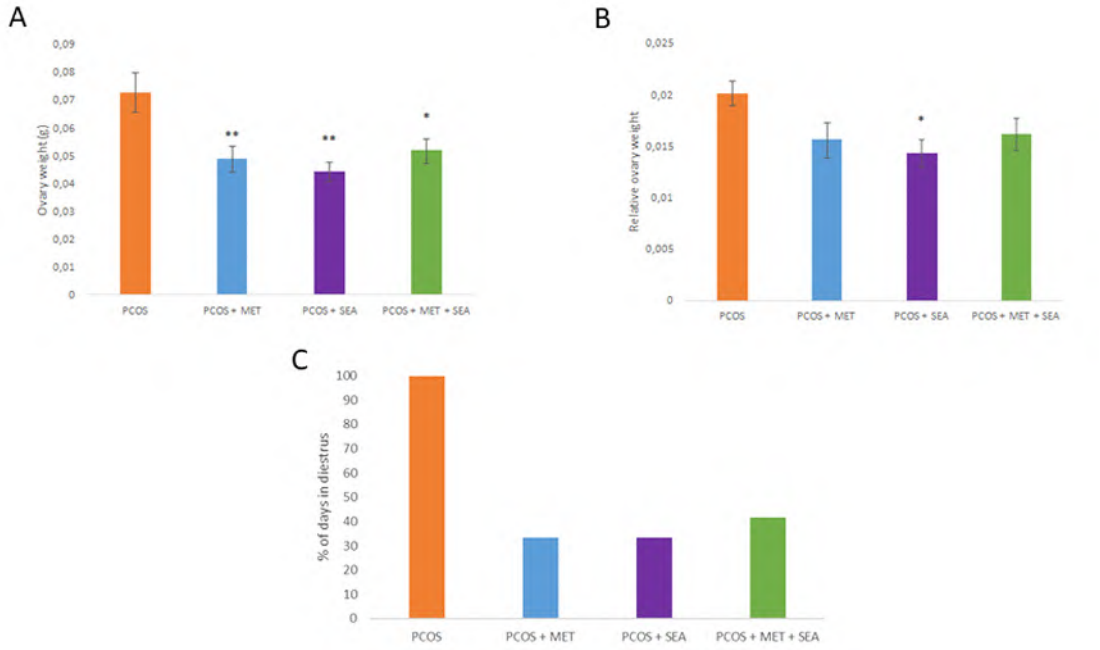
Ovary weight in groups treated with metformin ($p<0.01$) and Aronia melanocarpa extract ($p<0.01$), as well as in the group with a combined treatment of metformin and Aronia melanocarpa extract ($p<0.05$) was statistically significantly lower compared to the group treated with distilled water (Figure 2A).

The relative ovary weight was statistically significantly lower after the treatment with Aronia melanocarpa extract ($p<0.05$) compared to the non-treated group (PCOS). No statistically significant difference was registered between the other groups ($p>0.05$). Treatment with metformin alone, as well as in combination with Aronia melanocarpa extract did not cause a change in the relative ovary weight in animals with PCOS (Figure 2B).

PCOS group had 100% of days in diestrus, while the PCOS + MET group and the PCOS + SEA group had 33.33% of days in diestrus, and PCOS + MET + SEA group had 41.67% of the days diestrus during the analyzed period of 12 days (Figure 2C).

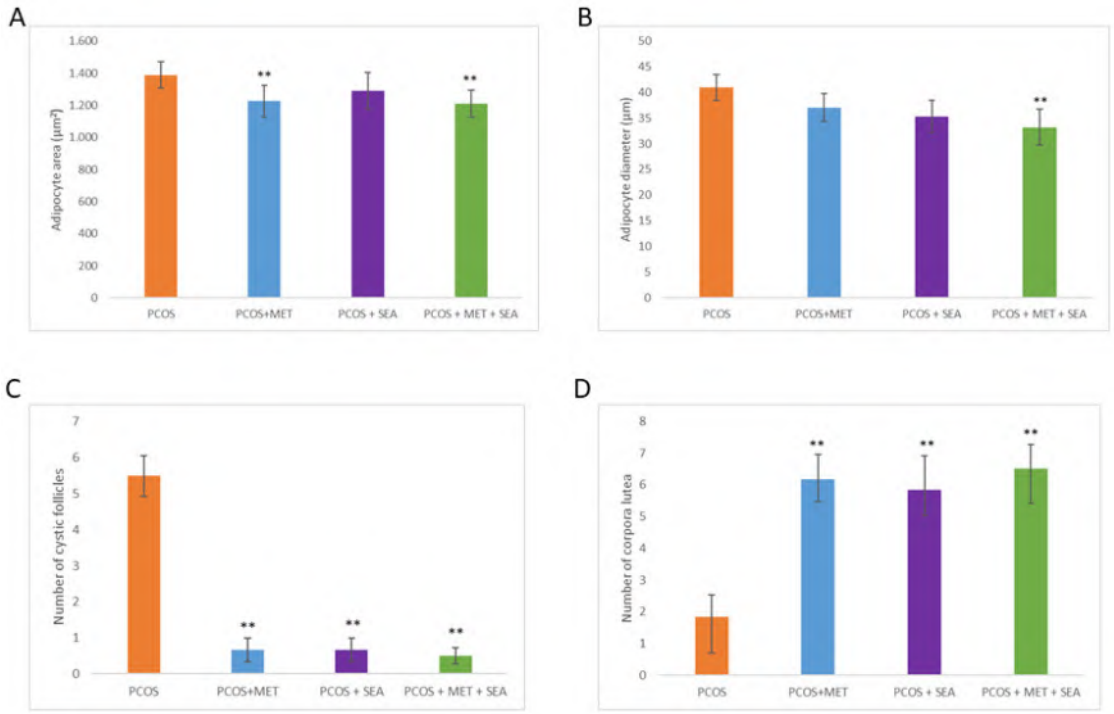
Adipocyte area was significantly decreased after treatment with MET as well as after combined treatment (Figure 3A), while adipocyte diameter was decreased only after combined treatment, compared to PCOS group (Figure 3B). The number of cystic follicles was decreased after all three type of treatments, while the number of corpora lutea was increased (Figure 3C and 3D).

Figure 2. The influence of usage metformin and Aronia melanocarpa extract, as well as their combined treatment on ovary weight and estrus cycle.



A – ovary weight; ** Statistical significance at the level of $p < 0.01$ compared to PCOS group.

Figure 3. The influence of usage metformin and Aronia melanocarpa extract, as well as their combined treatment on morphometric parameters of adipocytes and ovary tissues.



A - adipocyte area; B – adipocytes diameter;
C - number of cystic follicles; D – number of corpora lutea
** Statistical significance at the level of $p < 0.01$ compared to PCOS group.

DISCUSSION

PCOS represents a very complex syndrome with still unclear basic mechanisms of etiopathogenesis. There are a plethora of health implications that have been associated with the diagnosis of PCOS, many of these constituting lifelong complications. Metabolic anomalies and their associated manifestations are some of the most common risks. The treatment of this disorder is primarily symptomatic. Since none therapeutic approach has been established yet, the aim of many researches is to discover an alternative treatment approach as well as to provide new strategies that would result with a less side effects.

The present study investigated the effects of SEA, MET, or their combination on morphometric parameters and ovarian histological structure of the DHEA-induced PCOS rat model. Our results strongly confirmed the role of SEA alone or in combination with MET in alleviating disorders associated with PCOS. Statistically significant effect was recorded on body weight, body weight gain, relative ovary weight and ovary weight. In addition, subcutaneous fat adipocyte area was significantly reduced after all three type of treatment, while adipocyte diameter was decreased only in combined treatment (MET+SEA). Opposite, no statistically significant difference was shown in the change of body length, BMI and body fat index.

A five-week DHEA treatment successfully induced PCOS in rats. Following the method applied like Kim et al has produced very similar characteristics like those in patients with PCOS (29). All rats from the PCOS group were in the constant diestrus phase, acyclic during the last 12 days of the PCOS induction protocol (30). The rats exhibited a noteworthy recuperation of their estrous cycle, which now spanned a period of 4 to 5 days, subsequent to the administration of MET. Notably, MET is a widely recognized medication employed for ovulation induction, and has demonstrated efficacy in reinstating regular menstrual cycles in women (31). While no prior studies have been published regarding the utilization of Aronia Melanocarpa extract in animal models or human subjects, our investigation yielded compelling evidence. We observed that the SEA, when administered either in isolation or in combination with MET, exhibited a promising potential in reestablishing cyclicity within the treated groups. This intriguing finding suggests that Aronia Melanocarpa extract may hold substantial clinical promise as an adjunctive therapeutic agent in the management of menstrual irregularities. Further exploration is warranted to elucidate the underlying mechanisms and optimize dosage regimens for maximal efficacy in human patients. Additionally, clinical trials involving human subjects are imperative to validate these preliminary results and ascertain the safety and efficacy profile of this combined intervention. Such research endeavors could pave the way for a novel, integrated approach to address ovulatory dysfunction and menstrual irregularities, potentially offering renewed hope for individuals struggling with these challenges.

In our results, it was shown that the PCOS model induced by DHEA in postpubertal rats imitates the PCOS phenotype in obese patients, which is similar to results of Peng et al. study (32). Even not all preclinical studies confirmed the PCOS had an effect on body weight gain, our results indicated that PCOS model caused by DHEA in postpubertal rats acts like phenotype PCOS in obese patients (33). Opposite, PCOS models induced in prepubertal animals using DHEA commonly resulted in unchanged body weight compared to groups without PCOS (31). In our investigation, the implementation of all three treatment modalities led to a notable reduction in body weight gain subsequent to the induction of PCOS, as compared to the final body weight observed in the untreated rat group. This aligns with recent findings demonstrating a comparable weight-reducing effect of MET in both women and animal models of metabolic syndrome (34, 35). Favorable impact of SEA administration on body weight loss in rats with metabolic syndrome, as well as in individuals with diabetes mellitus, further corroborated the potential therapeutic benefits of this intervention (36, 37).

Remarkably, our results revealed a significant decline in body weight among rats with PCOS following a four-week regimen of SEA treatment. Nevertheless, it is noteworthy to mention that the incorporation of polyphenols from aronia extract into human dietary practices for weight management remains a subject of debate within the clinical community. This discrepancy may arise from a multitude of factors, including the presence of various clinical or subclinical comorbidities, as well as variations in applied protocols, all of which may yield divergent outcomes (38, 39).

Building on this, animal studies have consistently affirmed the advantageous impact of polyphenols derived from aronia extract on body weight regulation. These effects are intricately linked with the modulation of key pathways associated with insulin signaling, adipogenesis, and inflammation, all of which hold significant promise in augmenting the therapeutic armamentarium against PCOS (40).

Environmental determinants, encompassing dietary habits and lifestyle choices, alongside genetic predispositions, wield a substantial influence in the pathogenesis of PCOS. Notably, obesity not only exacerbates pre-existing PCOS manifestations but also portends unfavorable treatment responses, underscoring the pivotal role of weight management in PCOS care (41). Furthermore, compelling evidence suggests that women diagnosed with PCOS face an elevated risk of miscarriage, particularly when their BMI surpasses the threshold of 25 kg/m², underscoring the profound interplay between metabolic factors and reproductive outcomes in this context (8). In our study, all three type of treatments reduced adipocyte area compared to PCOS group. Mounting evidence indicates that an excess of androgenic hormones augments the size of adipocytes in women afflicted with PCOS. This hypertrophy of adipocytes can potentially culminate in dysfunctional adipose tissue, and the conversion of hypertrophic

adipocytes into smaller counterparts could represent a viable approach for ameliorating both IR and obesity. Similar results were observed in recent study (42) . Authors found decreased adipocyte area after administration of flavonoid rhamnocitrin, as well as MET, in letrozole-induced PCOS. However, additional effect of MET and SEA were observed in our study by significant decrease of adipocyte diameter in combined treatment compared to PCOS, contrary to monotreatment.

Histomorphological examination demonstrated cystic expansion within the ovaries, accompanied by a diminished count of viable follicles and a reduction in the layers of granulosa cells in the PCOS rats. Contrary, lower number of cystic formations and larger number of corpora lutea were observed in all three type of treatments, with a greater modification in combine treatment. In addition to evaluating estrous cycle regularity, the histological examination of ovarian tissue provided valuable insights into the successful induction of PCOS. This was evident in the PCOS group, where multiple follicular cysts were observed along with a notable reduction in the count of corpora lutea, indicative of diminished ovulatory events. Furthermore, a diminished presence of mature CL, coupled with a scarcity of developing follicles characterized by thicker granulosa layers exhibiting intermittent detachment and cumulus mass, as well as an enlarged stromal region, collectively suggest an underlying ovarian dysfunction akin to that observed in PCOS (38). Notably, post-treatment assessment revealed a discernible enhancement in ovarian morphology subsequent to SEA and MET administration, either individually or in combination. This was underscored by a notable reduction in the number of cysts, the presence of follicles at varying stages of growth, and a heightened abundance of corpora lutea compared to the PCOS group. These findings unequivocally underscore the beneficial impacts of both administered protocols on the ovarian microstructure. Ovary weight was reduced after all three type of treatments, while relative ovary weight was reduced only after SEA treatment. This potential of SEA was attributed to it unique polyphenols-rich content (43), which could influence body weight as well as ovarian weight (44, 45). Furthermore, the increased relative ovarian weight may be caused by the formation of follicular cysts (46), our results confirmed that SEA could exert a beneficial effects in PCOS while reduced follicular cysts are associated with obesity (47). The accompanying photomicrographs vividly illustrate the restorative effects of the administered treatments, particularly in ameliorating the cystic appearance of the ovaries. While this represents just one facet of the complex presentation of PCOS, it stands as a pivotal element in a larger mosaic of interrelated findings that collectively contribute to the understanding of this multifaceted syndrome.

Our study highlights the significant impact of these interventions on morphometric parameters, indicating their potential to address obesity, a prevalent comorbidity in PCOS. Notably, the reduction in adipocyte size and the modulation of adipose tissue morphology suggest a potential avenue for ameliorating metabolic dysregulations associated with PCOS.

Furthermore, the comprehensive assessment of ovarian histology provides crucial insights into the efficacy of the treatments in restoring ovarian function. The decrease in cyst formation and the increase in corpora lutea following treatment indicate a potential normalization of ovarian structure and function. Obesity emerge a significant determinants in the pathogenesis of PCOS, underscoring the importance of weight management strategies in PCOS care. Additionally, the heightened risk of miscarriage in women with PCOS, especially those with elevated BMI, highlights the intricate interplay between metabolic factors and reproductive outcomes. These findings may provide as a foundation for future clinical studies on the administration of SAE to women with PCOS in order to assess the specific impact of this extract on the reproductive and metabolic profiles of this patient population, particularly in those with an obese PCOS phenotype.

CONCLUSION

In conclusion, this study illuminates critical facets of PCOS, a complex and multifaceted condition with implications spanning metabolic, reproductive, and overall health. Our findings underscore the potential of SEA and MET, either in isolation or in combination, as promising interventions for mitigating PCOS-related complications.

The successful induction of PCOS in rats through DHEA treatment closely mirrors clinical presentations in obese PCOS patients, affirming the relevance of this animal model in studying the syndrome. The restoration of estrous cycle regularity following MET administration aligns with its established role in ovulation induction, further emphasizing its therapeutic promise. Moreover, the observed efficacy of SEA, a previously underexplored intervention, in reinstating cyclicity warrants further investigation to elucidate underlying mechanisms and optimize dosage regimens for human applications. This study contributes valuable knowledge towards the understanding and potential treatment of PCOS. While our investigation primarily focused on specific aspects of PCOS, it is imperative to acknowledge that PCOS encompasses a broader spectrum of clinical manifestations. Future research endeavors should aim to comprehensively address the multifaceted nature of this syndrome, with a view towards developing integrated therapeutic approaches that offer renewed hope for individuals grappling with PCOS-related challenges. Ultimately, these findings hold the potential to significantly improve the quality of life for individuals affected by PCOS.

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CONFLICT OF INTEREST

The authors declare no confict of interest.

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DEPRESSION, ANXIETY AND STRESS DURING THE COVID-19 PANDEMIC AMONG SERBIAN UNIVERSITY STUDENTS

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ABSTRACT

Our study focused at measuring stress, anxiety, and depression among the population of university students in Serbia. The sample included 493 students from The Faculty of Mechanical Engineering and the Higher Medical School, Kraljevo, University of Kragujevac, Central Serbia. The electronic survey was completed in approximately 10 minutes. Data collection was conducted during September and October, 2022. The research instruments included: General Questionnaire (used to collect demographic and personal data before and during the COVID-19 pandemic) and Depression Anxiety Stress Scales (DASS-21). All statistical calculations were performed using the standard commercial, standard software package SPSS, version 18.0. 12.8% of students reported severe and very severe symptoms of depression. In 21.7% of cases, severe and very severe symptoms of anxiety were reported. 20.3% of students reported sever and very severe symptoms of stress. Even though there are several studies on the mental health of Serbian college and university students during the COVID-19 pandemic, our article is unique in that it observes their mental health two years after the onset of the pandemic. This allows us to compare the findings with those obtained for the onset of the pandemic.

Keywords:*Depression, anxiety, stress, university students, COVID -19 pandemic.*

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INTRODUCTION

In December 2019, the world public had to confront the novel disease, COVID-19, when a new virus, SARS-CoV-2, was detected in the Chinese province of Hubei, in the city of Wuhan. To the prevent transmission of the infection COVID-19, the countries worldwide had to implement the recommended epidemiological measures: i.e. the restrictions in gatherings and exercise, social distancing, the lock-downs of social-life facilities, online learning and teaching, and working from home (1). Psychological studies have been emphasizing the general concerns about the mental health, especially in students, who had to face a sudden transition to online learning systems with limited resources (2). This transition brought the difficulties in communicating with professors, the lack of contact and support from peers, the difficulties in obtaining literature, and many other stressful changes; which have been all recognized as the potential sources of significant psychological issues (3). Student mental health during the pandemic has received considerable research attention, and numerous studies have been published worldwide on various aspects of mental health. Reportedly, student mental health deteriorated during the pandemic with varying levels of mental disorders, mainly anxiety, stress, and depression (4-8). Psychological stress, caused by social distancing and reduced social contacts, quarantine regulations, financial worries, frustration, boredom, lack of supplies, and poor communication, led to anger, confusion, anxiety, and depression (9-12). Physical distancing, as one of the most common measures, helped break the chain of infection transmission, but, on the other hand, it had a number of negative psychological effects, such as worries, fears, anxieties, and even the emergence of new mental illnesses (13,14). The findings of several studies conducted in Serbia testify to that. One study aimed at analyzing the psychological responses to COVID-19 pandemic in terms of perceived stress and related factors in the student population of the southeast Serbia. The study was conducted during the increased incidence of COVID-19 and the mean score of perceived stress amounted to 20.43 (± 7.67) (15). Another study was conducted with 580 undergraduate students of medicine at the University of Belgrade during the school year 2020 – 2021. It used Depression Anxiety Stress Scales (DASS-21) and showed that two thirds of the students who participated (age ranging from 21 to 30) reported symptoms of depression, extremely severe forms of anxiety, and severe stress (16). The vulnerability of student population and the deterioration of mental health in this population have been detected in many other countries. One study focused examined the prevalence and predictors of mental health disorders in 2349 students in May and June, 2020; in Poland, Slovenia, Czech Republic, Ukraine, Russia, Germany, Turkey, Israel, and Columbia. The prevalence of severe stress, depression, and generalized anxiety was 61.3%, 40.3%, and 30%, respectively (17).

Even though there are a few studies that were dedicated to measuring the levels of stress, anxiety, and depression of the student population in Serbia two years after the onset of the pandemic. Similar studies were conducted, but during the early stages. Our findings can be compared with those obtained earlier and as such, they can provide an insight into the potential changes during the different stages of the pandemic.

MATERIALS AND METHODS

The study was conducted as a cross-sectional study during September and October 2022. Since this was the beginning of the given shool year, the students were not additionally burdened by tests and exams. It is also important to note that Serbian educational system had already abandoned the online learning regime and students attended their classes regularly. The sample included 493 students from The Faculty of Mechanical Engineering and the Higher Medical School, Kraljevo, University of Kragujevac, Central Serbia. One medical and one non-medical educational profile were selected on purpose, due to our expectations that health-care students might be more willing to provide honest and precise answers. It is also important to highlight that higher medical schools in Serbian educational system do not provide study programs in medicine; they offer additional three-year education in health-care and nursing and can be enrolled after the highschool graduation.

Random sampling was used as the sample selection method. A one-stage sample was formed based on the percentage of students at the given faculties. The sample included the students from all years of study and of both genders. The deans of both faculties were informed in writing about the purpose and method of the survey. They both gave written consents for the survey to be conducted. The ten-minute survey was conducted electronically. It was completely voluntary and anonymous. The response rate was 86%. The respondents provided informed consents after they had been informed about the methodology and the purpose of this study on the first page of the electronic platform used to conduct the survey. The data were treated as highly confidential and were used for research purposes only. The questions that might identify the respondents were avoided. All necessary steps were taken, in accordance with the General Regulation for the Protection of Personal Data, the legislation of the Republic of Serbia, the European Legal Framework, the National Data Protection Act, the Strategy for the Protection of Personal Data, and the Law on Official Statistics Act, in order to protect the privacy and ensure the confidentiality of the data.

The research instruments were linguistically and culturally validated questionnaires in Serbian language. General Questionnaire was used to collect demographic and personal data about students' lives before and during the COVID-19 pandemic. It was used to collect the data on gender, age, type of settlement, faculty, and year of study. In addition, it included the questions inquiring whether the students felt endangered during the pandemic and what the reasons were for them to feel threatened: the fear of getting infected, the fear of endangering family members, the fear that close people (family, friends, etc.) may get infected, the fear of hospitalization, etc.

The standardized Serbian version of Depression Anxiety Stress Scales (DASS-21) was used to measure the levels of depression, anxiety, and stress (18). The questionnaire consists of 21 questions and three subscales which aim at evaluating the levels of depression, anxiety, and stress. The DASS-21 set comprises 3 subscales, with 7 questions per scale which are designed to evaluate the levels of depression, anxiety, and stress during the week prior to the survey. The *Depression* subscale focuses on the basic symptoms of depression: low positive affect, dysphoria, hopelessness, devaluation of life, self-deprecation, lack of interest and commitment, anhedonia, and sluggishness. The somatic symptoms present in depressive episodes, according to DSM-IV, such as sleep, appetite, and concentration problems, are excluded from this subscale since they are not specific for depressive disorders only, but also to anxiety disorders. The *Anxiety* subscale focuses on the symptoms of physiological arousal (e.g. dry mouth, breathing difficulties) and the effects on skeletal muscles (tremors), situational anxiety, and the subjective feeling of anxious affect. The *Stress* subscale aims at evaluating the symptoms of general, non-specific arousal, such as difficulty relaxing, nervous excitement, easy excitability and agitation, irritability, hypersensitivity, and impatience. Respondents rate how they have felt during the past week on a 4-point Likert scale, i.e., how strongly/frequently they experienced symptoms of depression, anxiety, and stress, from 0 ("not at all") to 3 ("most of the time or almost always"). Depression, anxiety, and stress scores are determined by summing the scores which can range from 0 – 21 for each subscale. The five categories (Normal, Mild, Moderate, Severe, Extremely Severe) are determined based on the scores, as follows: (1) depression: Normal (0 – 4), Mild (5 – 6), Moderate (7 – 10), Severe (11 – 13), Extremely Severe (≥ 14); (2) anxiety: Normal (0 – 3), Mild (4 – 5), Moderate (6 – 7), Severe (8 – 9), Extremely Severe (≥ 10); and (3) stress: Normal (0 – 7), Mild (8 – 9), Moderate (10 – 12), Severe (13 – 16), Extremely Severe (≥ 17). Extremely severe symptomatology is present if depression score is 14+, anxiety score is 10+, and stress score is 17+. These scores indicate the severity of symptoms, not the degrees of mental disorders (19,20).

The variables taken into consideration here include: (1) Demographic characteristics (gender, age, type of settlement, type of faculty, year of study), (2) Characteristics of life and study during the pandemic COVID -19, and (3) Mental health: i.e. depression, anxiety, stress.

Chi-square ($\chi 2$) test was used to compare differences in the frequency of categorical variables. The correlations between the dependent variables and a set of independent variables were examined by multivariate logistic regression. The risk was assessed by OR (odds ratio) size with a 95% confidence interval. All results with a probability of less than 5% ($p < 0.05$) were considered statistically significant. All statistical calculations were performed with the standard commercial, standard software package SPSS, version 18.0. (The Statistical Package for Social Sciences software (SPSS Inc, version 18.0, Chicago, IL)).

RESULTS

Socio-demographic characteristics of the student population

493 students participated in the study, 16.6% of which were from the Faculty of Mechanical and Civil Engineering and 76.7% of which were from the Faculty of Higher Vocational Studies. 23.3% of the respondents were male and 76.7% female. The average age of the population was 25.4 ± 8.3 years, with female subjects (26.43 ± 8.8 years) being significantly older than male subjects (22.6 ± 56 years) ($p < 0.001$). The largest percentage of the respondents belonged to the age group 18 – 24. Two-thirds of the respondents lived in urban areas (70.8%). Most students were on the first (34.7%) and third year of their studies (33.1%). The highest percentage of students reported living with their family (84.8%), followed by those living with their partner (7.3%).

Depression

The analysis focused on the symptoms of depression, revealed no significant difference between male and female subjects (p=0.985). The severe depression was found in 7.8% of men and 6.6% of women, while extremely severe depression was found in 5.2% of men and 6.1% of women. There was also no significant difference in the average scores between male (4.31±4.61) and female respondents (4.32±4.7), (p=0.985). The ANOVA test showed that there was no significant difference (p=0.379) between the mean depression scores in terms of age. No significant differences were found between different age groups (r=0.132). On the other hand, settlement typed appeared to be relevant. Severe and extremely severe forms of depression were more common in rural areas (11.8% and 7.6%, respectively) than in urban areas (4.9% and 5.2%, respectively), while moderate depression was ten times more common in urban settlements (11.7%) than in rural settlements (1.1%) (r=0.036). There was also a significant difference in the mean scores of near-depression between urban (4.02±4.51) and rural settlements (5.05±5.06) (p=0.027). There were no significant differences in either depression scores (p=0.540) or mean scores (p=0.611) with respect to the year of study (Table 1).

Table 1. Prevalence of depression in relation to sociodemographic characteristics of the student population

Variables	Without symptom		Mild depression		Moderate depression		Severe depression		Very severe depression		Average score	p
	n	%	n	%	n	%	n	%	n	%		
Gender												
Total	321	65.1	52	10.5	57	11.6	34	6.9	29	5.9	4.32±4.67	
Female gender	247	65.3	40	10.6	43	11.4	25	6.6	23	6.1	4.32±4.73	0.985
Male gender	74	64.3	12	10.4	14	12.2	9	7.8	6	5.2	4.31±4.61	
Age groups												
18-24	207	62.5	31	9.4	38	11.5	29	8.8	26	7.9	4.80±5.17	
25-29	28	66.7	6	14.3	6	14.3	1	2.4	1	2.4	3.45±3.65	
30-34	20	60.7	7	23.3	3	10.0	0	0.0	0	0.0	2.97±2.76	0.132
35-39	26	74.3	2	5.7	3	8.6	2	5.7	2	25.7	3.49±4.06	
40±	40	72.7	6	10.9	7	12.7	2	3.6	0	0.0	3.35±2.90	
Type of settlement												
Total	32	65.1	52	10.5	57	11.6	34	6.9	29	5.9	4.32±4.67	
Village	83	57.6	17	11.8	16	1.1	17	11.8	11	7.6	5.05±5.06	0.036
Urban	238	68.2	35	10.0	41	11.7	17	4.9	18	5.2	4.02±4.51	
Year of study												
First year	115	67.3	14	8.2	19	11.1	10	5.8	13	7.6	4.25±4.55	
Second year	88	62.9	22	15.7	16	11.4	8	5.7	6	4.3	4.13±4.51	
Third year	108	66.3	14	8.6	18	11.0	14	8.6	9	5.5	4.25±4.55	0.454
Fourth year	4	36.4	2	18.2	2	18.2	2	18.2	1	9.1	6.36±4.34	
Fifth year	6	75.0	0	0.0	2	25.0	0	0.0	0	0.0	2.75±3.15	

Anxiety

The findings demonstrate that there was a statistically significant difference in the mean values of the anxiety scores between male (3.17±3.58) and female respondents (5.00±4.66) (p=0.000). The female students were almost three times more likely to have extremely severe (13.5%:4.3%) and severe anxiety symptoms (12.2%: 4.3%). On the other hand, male respondents were seven times more likely (49.7%) to have mild symptoms (20%: 3.3%) and no symptoms of anxiety (63.5%) compared to female students (49,7%), (p=0.001). The students who reported living in rural areas had higher mean scores (5.07±4.63) compared to those living in urban areas (4.37±4.43). However, the T-test showed that this difference is not statistically significant (p=0.115).

There was also no significant difference in anxiety scores between the studied groups (p=0.383). The ANOVA test showed that there was a significant difference (p=0.001) between the mean scores among different age gruops. The mean scores decreased with age; in the youngest age group, the score was 4.89±4.79, while in the 35 – 39 age group, the mean score was significantly lower (-3.23±3.01). Although younger age groups have a higher prevalence of extremely severe and severe anxiety compared to other age groups, the $\chi 2$ test revealed no significant differences in the prevalence of different levels of anxiety among students with respect to age (p=0.390). No significant differences in either anxiety scores (p=0.776) or mean scores (p=0.426) was detected for different years of study (Table 2).

Table 2. Prevalence of anxiety according to sociodemographic characteristics of the student population

Variables	Without symptom		Mild anxiety		Moderate anxiety		Severe anxiety		Very severe anxiety		Average score	p
	n	%	n	%	n	%	n	%	n	%		
Gender												
Total	621	52.9	73	14.8	52	10,5	51	10.3	56	11.4	4.57±4.45	
Female gender	188	49.7	50	3.3	43	11.4	46	12.2	51	13.5	5.00±4.66	0.001
Male gender	73	63.5	23	20.0	9	7.8	5	4.3	5	4.3	3.17±3.58	

Variables	Without symptom		Mild anxiety		Moderate anxiety		Severe anxiety		Very severe anxiety		Average score	p
	n	%	n	%	n	%	n	%	n	%		
Age groups												
18-24	173	52.3	41	12.4	33	10.0	37	11.2	47	14.2	4.89±4.79	
25-29	21	50.0	11	26.2	5	11.9	2	4.8	3	7.1	4.52±4.43	
30-34	15	50.0	5	16.7	5	16.7	3	10.0	2	6.7	4.10±3.82	0.383
35-39	22	62.9	6	17.1	3	8.6	3	8,6	1	2.9	3.23±3.01	
40±	30	54.5	10	18.2	6	10.9	6	10.9	3	5.5	3.80±3.63	
Type of settlement												
Total	621	52.9	73	14.8	52	10.5	51	10,3	56	11.4	4.57±4.45	
Village	69	47.9	24	16.7	13	9.0	17	11.8	21	14.6	5.07±4.63	0.390
Urban	192	5.0	49	14.0	39	11.2	34	9.7	35	10.0	4.37±4.43	
Year of study												
First year	85	49.7	28	16.4	19	11.1	17	9.9	22	12.9	4.84±4.69	
Second year	81	57.9	15	10.7	12	8.6	16	11.4	16	11.4	4.44±4.41	
Third year	84	51.5	25	15.3	20	12.3	18	11.0	16	9.8	4.45±4.43	0.766
Fourth year	5	45.5	3	27.3	1	9.1	0	0.0	2	18.2	5.45±8.10	
Fifth year	6	75.0	2	25.0	0	0.0	0	0.0	0	0.0	2.38±2.13	

Stress

When it comes to stress, the findings reveal that there was a statistically significant difference in the mean scores obtained for male (6.58±4.61) and female respondents (8.46±5.22) (p=0.001). Females reported the symptoms of severe (14.6%) and extremely severe stress (8.7%) more often than males (7.5% and 3.5%, respectively) (p=0.015). The prevalence of stress in the student population decreases with age. The highest prevalence of stress was found in the younger age group, i.e., 18 – 24-year-olds, with one in ten students (10%) reporting extremely severe stress and one in six students reporting severe stress (15.7%).The lowest prevalence was found among respondents aged 40 and older (this difference is statistically significant (p=0.015)).

The ANOVA test showed that there was a significant difference (p=0.009) in the mean values of the total stress score between the youngest (8.60±5.49) and the oldest students (6.82±4.04). No significant differences were found in the prevalence of the various stress levels among students in relation to the type of settlement (p=0.130), but T-test revealed a statistically significant difference in mean scores between urban and rural areas (p=0.030). Students who reported living in rural areas had higher scores (8.81±5.45) compared to those who lived in urban areas (7.70±4.99). The analysis showed that there were no significant differences in either stress scores (p=0.408) or in mean score values (p=0.392) in relation to the year of study (Table 3).

Table 3. Prevalence of stress according to socio-demographic characteristics of the student population

Variables	Without symptom		Mild stress		Moderate stress		Severe stress		Very severe stress		Average score	p
	n	%	n	%	n	%	n	%	n	%		
Gender												
Total	275	55.8	50	10.1	68	13.8	63	12.8	37	7.5	8.02±5.15	
Female gender	199	52.6	42	11.1	49	13.0	55	14.6	33	8.7	8.46±5.22	0.015
Male gender	76	66.1	8	7.0	19	16.5	8	7.0	4	3.5	6.58±4.64	
Age groups												
18-24	168	50.8	29	8.8	49	14.8	52	15.7	33	10.0	8.60±5.49	
25-29	28	66.7	2	4.8	5	11.9	4	9.5	3	7.1	7.31±4.84	
30-34	22	73.7	3	10.0	3	10.0	2	6.7	0	0.0	6.33±3.78	0.015
35-39	21	60.6	7	20.0	4	11.4	3	8.6	0	0.0	6.74±3.81	
40±	36	65.6	9	16.4	7	12.7	2	3.6	1	1.8	6,82±4,04	
Type of settlement												
Total	275	55.8	50	10,1	68	13.8	63	12.8	37	7.5	8.02±5.15	
Village	76	52.8	10	6,9	19	13.2	25	17.4	14	9.7	8.81±5.45	0.130
Urban	199	57.0	40	11,5	49	14.0	38	10.9	23	6.6	7.70±4.99	
Year of study												

Variables	Without symptom		Mild stress		Moderate stress		Severe stress		Very severe stress		Average score	p
	n	%	n	%	n	%	n	%	n	%		
First year	93	54.4	17	9,9	21	12.3	26	15.2	14	8.2	8.27±5.29	
Second year	78	55.7	20	14.3	14	10.0	20	14.3	8	5.7	7.78±5.05	
Third year	93	57.1	12	74.0	31	19.0	14	8.6	13	8.0	7.95±5.05	0.408
Fourth year	6	54.5	0	0.0	1	9.1	2	18.2	2	18.2	10.0±5.71	
Fifth year	5	62.5	1	12.5	1	12.5	1	12.5	0	0.0	5.63±5.01	

Multivariate logistic regression (depression, anxiety, stress)

Multivariate logistic regression identified gender and age as the most important predictors of stress in the student population, as did the presence of anxiety and depression symptoms. Females were twice more likely to report stress (OR=2.106) than male respondents. The students in the youngest age group (18 – 24 years old) were three times more likely (OR=3.068) to report stress than students who were 40 years and older. Students who had symptoms of anxiety and depression were eight times and five times more likely (OR=8.189 and OR=5.364, respectively) to also report the symptoms of stress.

The most important predictor of anxiety was the fear of death. Students who were afraid of dying were 2.5 times more likely to exhibit symptoms of anxiety (OR=2.492). The association with other mental disorders, namely stress (OR=7.913) and depression (OR=5.520) was also significant in this model. Females were twice more likely to exhibit depression symptoms than male subjects (OR=1.997). Students who exhibited symptoms of anxiety (OR=5.309) and stress (OR=5.634) were five times more likely to also have depression (Table 4).

Table 4. Cross-over odds ratios (OR) and 95% confidence intervals (CI) for stress, anxiety, depression and selected variables

Variable	Category	Stress			Anxiety			Depression		
		OR	95%CI	p	OR	95%CI	p	OR	95%CI	p
Gender	Female	2.106	1.135-3.909	**0.018**	1.373	0.748-2.528	0.309	1.997	1.063-3,751	**0,032**
	Male	1			1			1		
Age groups	18-24	3.068	1.28-7.340	**0.012**	0.693	0.304-1.580	0.384	1.21	0.511-2.862	0.664
	25-29	0.836	0.255-2.743	0.768	1413	0.481-4.150	0.529	1.216	0.373-3.965	0.745
	30-34	0.505	0.148-1.721	0.275	1.200	0.397-3.630	0.747	1.741	0.526-5.956	0.560
	35-39	1.623	0.513-5.133	0.410	0468	0.142-1.563	0.211	0.815	0.246-2.70	0.738
	40+	1			1			1		
Faculty*	1	0.698	0.310-1.574	0.387	0.577	0.342-1.678	0.493	0.577	0.342-1.678	0.493
	2	1			1			1		
Year of study	First	0.23	0.026-2.037	0.187	3.000	0.361-24.908	0.309	1.154	0.147-9.084	0.892
	Second	0.278	0.033-2.352	0.240	1.717	0.211-13.961	0.613	1.742	0.220-13.974	0.598
	Third	0.248	0.029-2.141	0.205	2.482	0.305-20.206	0.316	1.278	0.158-0.965	0.845
	Fourth	0.104	0.007-1.573	0.103	3.645	0.291-45.522	0.430	3.645	0.291-45.522	0.430
	Fifth	1			1			1		
Type of settlement	Urban	1.126	0.644-1.967	0.677	1.226	0.744-2.167	0.576	0.383	0.395-1.180	0.576
	Village	1			1			0.171		
Anexiety	Yes	8.189	4.784-14.016	0.001				5.309	3.006-9.376	0.001
	No	1						1		
Depression	Yes	5.364	3.017-9.537	0.001	5.52	3.074-9.940	0.001			

Variable	Category	Stress			Anxiety			Depression		
		OR	95%CI	p	OR	95%CI	p	OR	95%CI	p
	No	1			1					
Stress	Yes				7.913	4.651-13.463	0.001	5.634	3.232-9.822	0.001
	No				1			1		

*1-Engineering and construction 2 - Higher vocational schoo

DISCUSSION

The pandemic COVID-19 quickly became one of the largest global crises. It has had serious and far-reaching consequences on health systems, economies, and societies. The changed contexts of education, work, movement, gatherings, behavior, leisure activities, life with family and partners, have certainly affected almost the entire global population. The fear of the unknown during the pandemic has negatively affected all areas of life, including the mental health of children and adults (1). Although our study was conducted two years after the onset of the pandemic, the findings are alarming. The symptoms of depression were reported by 34.9%, of anxiety by 47.1% and of stress by 44.2%. Severe and extremely severe depression, anxiety and stress scores were detected in 12.8%, 21.7%, and 20.3% of participants, respectively. However, if these results are compared to the findings of one study conducted at the beginning of the pandemic, we can observe that levels of stress, depression, and anxiety declined. Reportedly, at the onset of the pandemic, 64.5% of students reported the symptoms of depression, 66.8% severe levels of anxiety, and 66.7% extremely severe symptoms of stress (15). These results may suggest that students adapted to new circumstances and developed coping mechanisms.

Longitudinal studies comparing mental health before and during the pandemic are scarce. One study with 254 students in the UK revealed a significant increase in depression and a decrease in well-being during the first lockdown (April/May 2020) compared to the state of psychological well-being prior to the pandemic. The authors found that over a third of the participants were clinically depressed at the time of isolation, i.e. there had been an increase of 15% in comparison to the levels prior to the pandemic (21). Another longitudinal study of 214 UK university students found a decline in levels of mental well-being and an increase in levels of perceived stress during the first lock-down (22).

The study conducted with the students of Higher Healthcare Vocational School in Belgrade before the pandemic used DASS-42. Their findings indicate that depression, anxiety, and stress were present in 13.6%, 25.6%, and 26% of students, respectively. These values are significantly lower than those reported by the studies conducted during the pandemic (16,17) but also lower than the values obtained through our study. This clearly indicates that the pandemic has had a significant impact on the mental health of the student population.

A study on the mental health under the "COVID -19 measures" revealed a very prominent presence of moderate to severe depression (48%), anxiety (about 38%) and suicidal thoughts (18%). No less than 71% of the respondents reported an increase in stress and anxiety during the pandemic, while less than half of the respondents (about 43%) reported being able to adequately manage stress (23). Study by Ma et al. included more than 700,000 Chinese students and it found that nearly 45% of their cohort suffered from mental health problems, with anxiety being the most common symptom (7). A study by Chen and Lucock included 1173 undergraduate and postgraduate students at one university in the UK. They found that more than 50% of the subjects had anxiety and depression levels above the clinical borderline level (24). Similarly, a multinational study conducted among college students in nine countries found a high prevalence of stress (61.3%), depression (40.3%), and anxiety (30%) (25).

Large survey studies of the mental health in college students conducted in the UK have found high levels of anxiety and depression, increased sedentary behavior, and poor quality of sleep (26,27).

Our results showed, when it comes to stress, that there was a statistically significant difference in the mean scores obtained for male and female respondents . Females reported the symptoms of severe (14.6%) and extremely severe stress (8.7%) more often than males (7.5% and 3.5%, respectively). The prevalence of stress in the student population decreases with age. No significant differences were found in the prevalence of the various stress levels among students in relation to the type of settlement and in relation to the year of study.

The female students were almost three times more likely to have extremely severe (13.5%:4.3%) and severe anxiety symptoms (12.2%: 4.3%). On the other hand, male respondents were seven times more likely (49.7%) to have mild symptoms (20%: 3.3%) and no symptoms of anxiety (63.5%) compared to female students (49,7%). Severe and extremely severe forms of depression were more common in rural areas (11.8% and 7.6%, respectively) than in urban areas (4.9% and 5.2%, respectively), while moderate depression was ten times more common in urban settlements (11.7%) than in rural settlements (1.1%). And other studies have shown similar results to ours (16,17). Contrary to our results, some studies have shown that older students have a higher frequency of depressive symptoms (28). Some of the reasons for older students to be more psychologically burdened compared to

younger ones may include: uncertainty of their future after the graduation, concerns about finding employment, financial uncertainty, the expectations of the environment that a young person of this age should be accomplished in significant social roles such as getting married and having offspring (29-31).

The pandemic has changed the conditions of our everyday life. The full impact of these changes and all consequences are yet to emerge. Hence, the outcomes still cannot be fully understood. Due to the rapid spread of the virus and its negative effects on mental health, the importance of developing adequate programs for the prevention of mental disorders in student population must be highlighted. The results of this study testify to the fact mental health of the youth should be monitored through consistent and comprehensive research.

This study is unique in that we aim at measuring stress, anxiety, and depression among students two years after the onset of the pandemic. Our findings can be compared with those obtained earlier and as such, they can provide an insight into the potential changes during the different stages of the pandemic. However, our study has certain limitations related to the coverage of the student population. Students of different profiles and from different regions of Serbia should be included, which can be the subject of a subsequent study.

CONCLUSIONS

In order to create adequate public health policies and strategies that are needed to improve mental health and prevent mental disorders during the COVID-19 pandemic, it is essential to determine and expose different predictors of mental health. These findings can improve our future preparedness in case of other unexpected pandemic or disaster. Seen from a public health perspective, the promotion of mental health and the prevention of mental disorders in the student population is essential for achieving the progress of the whole society.

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INSTITUTIONAL REVIEW BOARD STATEMENT

Deans of the Faculty of Mechanical Engineering and the Higher Medical School, Kraljevo, University of Kragujevac, Central Serbia, gave written consents for the survey to be conducted.

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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UTILIZATION OF GONADOTROPIN-RELEASING HORMONE AGONISTS IN THE TREATMENT OF METASTATIC AND LOCALLY ADVANCED PROSTATE CANCER - COMPARISONS OF PRACTICES FROM THREE EUROPEAN COUNTRIES

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ABSTRACT

Introduction: Prostate cancer is one of the most common health threats for men in the developed world. With the advent of prostate cancer screening using serum prostate-specific antigen (PSA) tests, prostate cancer mortality has declined at the expense of substantial disease overtreatment. Modern prostate cancer therapy is performed according to certain guidelines. Anti-androgens are compounds that inhibit the action of androgens in prostate cancer cells by blocking receptors and preventing the binding of hormones to them. Aim: The aim of this research is to analyze the use of registered forms of LHRH agonists used in the treatment of locally advanced and metastatic prostate cancer in the last five years to examine trends in prescribing this group of drugs in Serbia whose patients gravitate towards the Urology Clinic at the UCC Kragujevac. Material and method: Using the ATC/DDD methodology, the use of LHRH agonists at the Urology Clinic of the UCC Kragujevac. A retrospective study of the use of this group of drugs according to the ATC classification was performed on the basis of data obtained from the hospital pharmacy for the period from year 2016 to year 2021, and the results are expressed by the number of DDD per 1000 inhabitants per day (1000/ inhabitants/day). In the observed period, 1361 patients with a diagnosis of C61 (malignant prostate tumor) were treated at the Clinic of Urology. Results: In the observed period, a preparation containing triptorelin in a dose sufficient for one month of therapy was most often used. The total consumption of gonadorelin was lower compared to the rest of Serbia and EU countries, which was expected due to the protocol and the number of patients who gravitated towards the UCC Kragujevac. Conclusion: Despite certain limitations, this evaluation represents the first attempt to summarize the available evidence on the prescribing of LHRH agonists in Serbia. It was found that the consumption in UCCKG is lower compared to the consumption of these drugs in Serbia, Croatia and Italy at the same time intervals, for the same observed diagnosis.

Keywords: *prostatic neoplasms; LHRH agonists, ATC/DDD metholology, drug utilization.*

INTRODUCTION

Prostate cancer is one of the main medical problems faced by the male population. It is one of the most common neoplasms in men, together with colorectal and bronchopulmonary cancer[1,2]. Modern prostate cancer therapy is performed according to certain guidelines. Certain treatment methods (monitoring, surgical, and non-surgical treatment) are used for each stage of the disease. Non-surgical treatment may involve radiation therapy, chemotherapy, hormone therapy, external beam radiation therapy, and particle therapy, high-intensity focused ultrasound, or some combination.[2]

Hormone therapy, which aims to reduce testosterone levels, is indicated by the European Urological Association as the first therapeutic option in patients with prostate cancer and metastatic disease.[3] Dependence of the activity od androgen hormons of prostate and seminal vesicles has been known for several centuries. Since prostate cancer originates from the adult epithelium of the prostate, Huggins and Hodges[4] in 1941 suggested that this cancer was dependent on the hormonal activity of androgens. Endocrine therapy has no curative effect. About 20-30% of prostate cancers do not respond to endocrine therapy, because the metabolism and activity of these cancer cells is independent of the activity of androgen hormons. Endocrine therapy can be divided into four groups:[5] androgen-producing organ ablation (surgical castration - orchiectomy), androgen synthesis inhibition (aminoglutetamide and ketoconazole), hypothalamic and pituitary suppression (gonadotropin-releasing hormone agonists (LHRH agonists) – pharmacological castration) and the inhibition of androgen activity in effector cells (anti-androgens, central and peripheral). Pharmacological blockade is achieved by the use of gonadotropin-releasing hormone (LHRH) agonists[6] and / or anti-androgens. Antiandrogens are compounds that inhibit the action of androgens in prostate cancer cells by blocking androgen receptors. Since testicular androgens are eliminated by surgical or pharmacological castration, the action of extrastratestic androgens is suppressed by the administration of various drugs.[7] By combining these two modalities of therapy, maximum or total androgen blockade is achieved.[8] Drug consumption analysis is a way of quality assurance and is used to monitor and evaluate the use of drugs according to agreed criteria/standards, and if necessary, it recommends a change in practice in order to improve the quality, safety and profitability of prescribed drugs.[9]

To monitor drug consumption, the World Health Organization (WHO) proposes methodology for anatomical-therapeutic-chemical classification of drugs (ATC) / defined daily dosage (DDD), where drug consumption is expressed as the number of DDD / 1000 inhabitants / day (DID). The ATC / DDD methodology makes it possible to standardize consumption monitoring and to overcome differences in the size of the population for which consumption is monitored. The ATC drugs (Medicinal Products) classification is an internationally accepted classification of drugs (Medicinal Products), where each International Nonproprietary Name (INN) of drug corresponds to a seven-character code divided into five levels of classification.[10]

The aim of this research is to analyze the use of registered forms of LHRH agonists used in the treatment of locally advanced and metastatic prostate cancer in the last five years to examine trends in prescribing this group of drugs in Serbia whose patients gravitate towards the Urology Clinic at the University Clinical Centre Kragujevac (UCCKG).

MATERIAL AND METHOD

Study design

The study was designed as a retrospective cross-sectional study. The study was conducted in two phases. In the first phase, data were collected on the consumption of LHRH agonists in the period from 2016 to 2021, by inspecting the list of consumption and procurement of drugs at the Urological Clinic of the UCC KG, and data on the number of patients by inspecting the medical histories of the clinic through the health information system. In the second phase, the trend of consumption of LHRH agonists was analyzed, after which the obtained results were compared with the same data for the territory of the Republic of Serbia and the surrounding countries. The analysis of secondary data on the consumption of LHRH agonists from the compared countries (Serbia, Italy, Croatia) was performed through annual reports available with the websites of the National Drug Agencies.[11,12,13,14,15]

Study population

In the observed period, 1361 patients with a diagnosis of C61 (malignant prostate tumor) were treated at the Clinic of Urology at the UCC Kragujevac. The introduction of LHRH agonists into therapy, a change in the form of the drug or a therapeutic approach is induced by the uro-oncological council of the UCC Kragujevac. The basic criteria for inclusion were patients who were admitted to the Urology Clinic under the diagnosis of malignant prostate tumor (code C61 according to the International Classification of Diseases (ICD)-10). The exclusion criteria were a change in treatment regimen or death during the use of LHRH agonists.

Drug utilization and data processing:

To analyze the consumption of LHRH agonists at the Clinic of Urology, the ATC / DDD (anatomical-therapeutic-chemical classification of drugs / defined daily dose) methodology was applied, where the drug consumption was calculated as the number of DDD / 1000 inhabitants / day. No dosage forms from the same ATC group such as buserelin and histrelin have been registered in the Republic of Serbia. According to the WHO ATC / DDD classification (World Health Organization), the DDD for leuprorelin for this dosage form is 0.134 mg, for goserelin 0.129 mg and for triptorelin 0.134 mg[11]. Data on the number of inhabitants in the region of Šumadija and Western Serbia are taken from the Statistical Yearbook of the Republic of Serbia for each

individual year.[11-15] ATC classification of drugs (Medicinal Products), which consumption is monitored, shown in Table 1, together with DDD and way of drug administration.

RESULTS

In the period from 2016 to 2020, the Urology Clinic of the UCCKG prescribed all three drugs listed in the ATC classification in the following dosage forms (Table 1). The introduction of LHRH agonists, as well as changed in drug formulation or therapeutic approach is indicated by uro-oncologic consilium of UCC Kragujevac.

The largest share in the number of defined daily doses in all observed years has a preparation containing triptorelin in a dose of 3.75 mg, which is sufficient for therapy for four weeks (Figure 1). Figure 2 presents the consumption of LHRH agonists depending on the formulation (monthly, quarterly and six-month). A preparation containing leuprorelin in a dose of 11.25 mg in the observed period was prescribed only in year 2016. From the end of 2019 and during the year 2020, a significant share in the number of DDD has the preparation of leuprorelin in a dose of 22.5 mg, a form intended for therapy lasting 6 months.

Table 3. shows the five-year consumption of LHRH agonists in Serbia, Croatia and Italy in relation to the consumption at the Clinic for Urology of the University Medical Center Kragujevac. Consumption is expressed in DDD/inhabitant/day, so it can be stated that consumption in UCC Kragujevac is quite lower compared to consumption in the rest of Serbia. At the same time, the total consumption in Serbia is quite higher in relation to Italy and Croatia,especially in the last observed year 2020.

DISCUSSION

Consumption of LHRH agonists shows a growth trend in the period from 2016 to 2021. This may be due to the increased survival rate of these patients based on current treatment guidelines. A 2020 study by Taiwanese authors from Kuang-Ming Liao et al. has confirmed this, especially when prostate cancer is detected early[24]. The Medical Research Council[25] conducted an important study regarding this problem, and on that occasion it was proven that early treatment of LHRH agonists improves the average survival by three months. In our study, different triptorelin formulations and forms for one-month therapy of all LHRH agonists were mostly used. Table 2 shows the five-year consumption of LHRH agonists in Serbia, Croatia, and Italy relative to the consumption in our study.

It can be stated that the consumption in UCC Kragujevac is quite lower in relation to the consumption in the rest of Serbia. A possible explanation is that although the UCCKG gravitates to almost 2 million people from the region of Šumadija and Western Serbia, a large number of these patients are treated in a local reference health institution or in specialized institutions for the treatment of oncological diseases in Belgrade. Therefore, the obtained consumption parameters are significantly lower compared to what would be realistic. Total consumption in Serbia is lower compared to Italy and Croatia, especially in 2019.

In all years, the use of triptorelin preparations in different doses is the most common. This can be explained by the results of the study by Myungsun Shim et all. from 2019, which show that triptorelin achieves the lowest mean testosterone level and the highest rate of chemical castration of testosterone (10ng / dl)[26]. During 2020, the use of leuprorelin preparations increased 10 times compared to the use from 2019, and 35 times compared to the use from 2018. This is also shown by the research conducted in Germany in 2015 by Axel Merseburger et all[27]., which compared consumption in Germany in relation to Great Britain, France, Italy and Sweden. The justified use of leuprorelin has shown the possibility of significant savings in this segment, which can be allocated to other elements of therapeutic strategies, including new therapeutic innovations.

In general, in the observed period, there is a growing trend in the use of drugs from the group of gonadorelin analogues. Unlike the urology clinic of the UCCKG and Serbia, triptorelin is most often used in Croatia and Italy. In Croatia[13], in the range from 2016 to 2019, the total consumption of the observed drugs increased by almost 100%, and only between 2018 and 2019, a difference of 28% appeared. Also, looking at the last year of this research(2019.) we notice that Croatia records higher consumption compared to Italy[14] (20%) while in comparison with Serbia, the total number of prescribed drugs from the observed group is almost 4 times higher in Serbia. A study from the year 2011. by S.Lannazo et all[28] in Italy showed that leuprorelin 22.5 mg was the most cost-effective option for selecting LHRH agonists in that country. The results of a multicenter study of drug use assessment in prostate cancer therapy in France, Germany, Italy, Spain and the United Kingdom[29] indicate a high and growing prevalence of prostate cancer in Europe. The reason for this is the aging of the population, which brings with it high costs. Therefore, it is necessary for the Ministry of Health to be aware of this disease as a priority disease. The results of a national population study in Sweden[30] suggest that a threefold increase in LHRH agonist use is associated with a moderate reduction in mortality in men between 65 and 74 years of age with newly diagnosed locally advanced prostate cancer. Recent advances in the formulation of pharmaceutical formulations in the treatment of prostate cancer represent a good opportunity to reduce the cost of therapy.[31]

The disadvantages of this study relate to the observed group of drugs, since the conciliatory decisions are made on the basis of the recommendations of the European Association of Urology[3] (European Association of Urology) so individual variations in prescribing are minimized. In addition, the constant supply of the hospital pharmacy with certain types of LHRH agonists can be an analytical problem in terms of constant use of the prescribed LHRH agonist.

Table 1. ATC classification of gonadorelina registered forms which is used for treatment od advent tumors prostate in Republic of Serbia

Mark	Clasification	DDD (1000/day)[23]	Method of administration/application
L	Antineoplastic and immunomodulators		
02	Endocrinological therapy		
A	Hormones and related drugs		
E	Gonadorelin-releasing hormone analogues		
02	Leuprorelin[16,17]	60 mcg	intramuscular / subcutaneous
03	Goserelin[18,19]	0,129 mg	subcutaneous
04	Triptorelin[20,21,22]	0,134 mg	intramuscular (i.m.)

Table 2. Overview of the five-year consumption of all LHRH agonists used, depending on the dose and formulation of the drug

International Nonproprietary Name – INN formulation	Proprietary name	Dosage	Pharmaceutical formulation	Consumption (DDD/1000/day)
leuprorelin (monthly)	Lupron® 3.75 mg/ml	3.75 mg/ml	Powder and solvent for suspension for injection in a pre-filled syringe	0,004357974
leuprorelin (q3-month)	Lupron® 11.25 mg/ ml	11.25 mg/ml	Powder and solvent for suspension for injection in a pre-filled syringe	0,001289482
leuprorelin (q3-month)	Lutrate depo® 22.5mg	22.5 mg	Powder and solvent for prolonged-release suspension for injection	0,019165
goserelin (monthly)	Zoladex®	3.6 mg	Implant, in pre-filled syringe	0,019552701
goserelin (q3-month)	Zoladex® LA	10.8 mg	Implant, in pre-filled syringe	0,002876386
triptorelin (monthly)	Diphereline® 3.75 mg/2 ml	3.75 mg/2 ml	Powder and solvent for prolonged-release suspension for injection	0,176707295
triptorelin (q3-month)	Diphereline® 11.25 mg/2 ml	11.25 mg/2 ml	Powder and solvent for prolonged-release suspension for injection	0,021587071
triptorelin (q6-month)	Diphereline® 22.5 mg/2 mL	22.5 mg/2 ml	Powder and solvent for prolonged-release suspension for injection	0,00096538

Table 3. Comparative data on the consumption of gonadorelin analogues at the Clinic of Urology of the University Medical Center Kragujevac, as well as in Serbia, Croatia and Italy by years.

International Nonproprietary Name/ region	Consumption of LHRH agonists in DDD / 1000 / day ** per year				
	2016	2017	2018	2019	2020
Clinic of Urology, UCC Kragujevac					
Leuprorelin	0,0020	0,0022	0,0005	0,0017	0,0184
Goserelin	0,0028	0,0037	0,0054	0,0070	0,0035
Triptorelin	0,0353	0,0316	0,0441	0,0489	0,0394

International Nonproprietary Name/ region	Consumption of LHRH agonists in DDD / 1000 / day ** per year				
	2016	2017	2018	2019	2020
Total	0,0402	0,0375	0,0500	0,0576	0,0613
Serbia[12]					
Leuprorelin	0,0563	0,0720	0,0014	0,0055	N/A *
Goserelin	0,1035	0,0841	0,0832	0,0821	N/A
Triptorelin	0,1927	0,2076	0,2706	0,2817	N/A
Total	0,3526	0,3637	0,3552	0,3694	N/A
Croatia[13]					
Leuprorelin	0,5700	0,5800	0,5900	0,7800	N/A
Goserelin	0,0900	0,1100	0,1300	0,1900	N/A
Triptorelin	0,0300	0,0300	0,2300	0,2300	N/A
Total	0,6900	0,7200	0,9500	1,2000	N/A
Italy[14]					
Leuprorelin	N/A	0,2000	0,2000	0,2000	N/A
Goserelin	N/A	0,0000	0,0000	0,0000	N/A
Triptorelin	N/A	0,7000	0,8000	0,8000	N/A
Total	N/A	0,9000	1,0000	1,0000	N/A

* N/A – data not available;
** DDD/1000/day – the number of consumed doses of LHRH agonists per 1000 inhabitants in the observed region per year.

Figure 1. Consumption of gonadorelin analogues by type of drug by years.

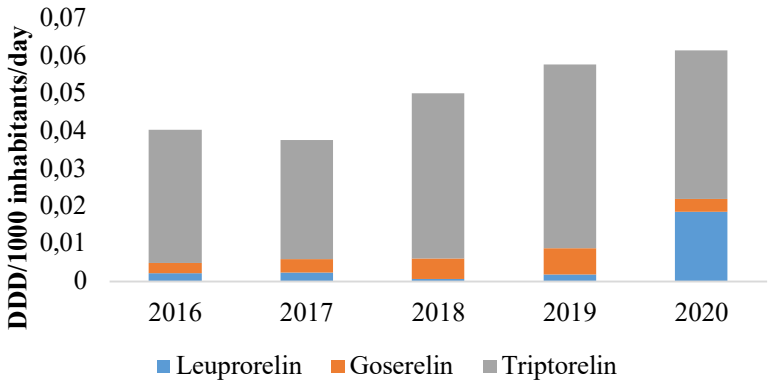
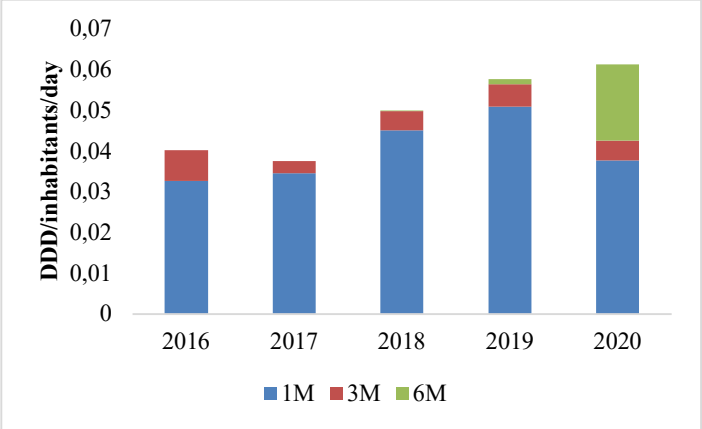


Figure 2. Consumption of gonadorelin analogues by formulation by year



CONCLUSION

It was found that the consumption is lower compared to the consumption of these drugs in Serbia, Croatia and Italy at the same time intervals, for the same observed diagnosis. Despite certain limitations, this evaluation is the first attempt to summarize the available evidence on prescribing LHRH agonists in Serbia. Such studies should provide guidance to medical doctors who should make rational choices regarding the use of LHRH agonists. Monitoring the consumption of drugs is useful from the clinical and pharmacoeconomic aspect and requires the engagement of several experts in this field.

CONFLICTS OF INTEREST

There is no conflict of interest among the signed authors.

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THE CHORIOAMNIONITIS - STILL AN ENIGMA IN PERINATOLOGY

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ABBREVIATIONS

PPROM - preterm premature rupture of amniotic membranes
AHCA - acute histological chorioamnionitis
IL1 – interleukin 1
IL6 – interleukin 6
IL8 – interleukin 8
IL10 – interleukin 10
TNF - tumor necrosis factor
CD8 TLy - cytotoxicity T lymphocytes
CXCL9 - C-X-C motif chemokine 9
CXCL10 - C-X-C motif chemokine 10
CXCL11 - C-X-C motif chemokine 11
HLA-G - human leukocyte antigen G (histocompatibility antigen, class I, G)
DNA - deoxyribonucleic acid

ABSTRACT

Chorioamnionitis is one of the most common causes of preterm premature rupture of fetal membranes and consequent preterm birth. The variety of mechanisms underlie pathophysiology of chorioamnionitis represents its greatest enigma. The unspecific clinical manifestations of chorioamnionitis considered an aggravating issue for perinatologist to diagnose it timely. There are no absolutely sensitive, non-invasive diagnostic procedures for certain establishment of chorioamnionitis diagnosis. The more sensitive diagnostic procedures are also invasive, so the question of their application in routine practice arises. Certainly, the perinatologist is always in a dilemma when there is a suspicion of chorioamnionitis, whether and how it should be treated, having in mind the facts about possible side effects on the fetus, but also on the mother. This paper presents a summary of all known facts about the etiopathogenesis, classification and clinical manifestations of chorioamnionitis, providing a basis for further research regarding the identification of more sensitive diagnostic markers, as well as the treatment of this condition.

Keywords: *Chorioamnionitis, preterm birth, inflammation, infection.*

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INTRODUCTION

Definition and classification of chorioamnionitis

The significance of the placenta, as a temporary organ, is defined by one simple sentence "the placenta represents the diary of gestational life" [1]. It reflects not only the intrauterine environment, but also provides valuable data related to maternal pathology [2,3]. As an extremely complex and multifunctional organ, placenta still remains insufficiently examined. It consists three main parts: the placental disc, chorioamniotic membrane and umbilical cord [4,5]. The terms placentitis and vilitis both represent the inflammation of placental disc (chorionic villi) [4,5], but reflect different stages of the same inflammatory process, therefore should not be considered as synonyms. On the other hand, funisitis is referred to inflammatory process that affects the umbilical cord [6,7].

Literally, the term chorioamnionitis refers to the inflammatory reaction that takes place in the fetal membranes (amnion and chorion) [8]. Chorioamniotic membranes form the most distant part of the conceptus [9]. On the one hand, they represent the boundary, while on the other hand they represent the connection between mother and fetus. The inflammation process can affect amnion or chorion exclusively; although the most common form represent the sublimation of the inflammatory process in both [8]. The recommendation of the US National Institutes of Health Conference is to switch autologous to the heterologous form of chorioamnionitis, which also includes inflammation of both decidua and amniotic fluid [8].

Based on the course severity, two form of chorioamnionitis can be distinguished: acute and chronic. Regarding the pathophysiological aspect, the chorioamnionitis could be defined as infectious or aseptic [4]. Another classification, regarding the absence or presence of clinical manifestations, could be arranged as clinical or asymptomatic [10,11], while some authors also considered histological, microbiological and biochemical form of chorioamnionitis as appropriate [12]. Apart from the classification into acute and chronic chorioamnionitis, all other classifications have pronounced deficiencies. However, there is no single consensus regarding classification of chorioamnionitis, due to imprecise and heterogeneous definitions [8], misunderstanding of complex molecular mechanisms and deficiency of taxonomy [13]. For example, histological chorioamnionitis (as the most common form) refers to not only clinical, but also asymptomatic, infectious, and aseptic chorioamnionitis [14].

Although the above mentioned classification is important for understanding the pathophysiology and manifestation of the clinical course of chorioamnionitis, in practical terms it is not simply applicable. Thus, histological chorioamnionitis can be of acute and chronic course, clinical chorioamnionitis can be infectious and aseptic, while any histological chorioamnionitis does not have to be clinically manifested, nor in the basis of infectious etiology.

Incidence of the chorioamnionitis

The estimated incidence of chorioamnionitis is 2-4% of term delivery [15,16] and 40-70% of preterm birth [15,17]. The frequency of acute chorioamnionitis is inversely proportional to gestational age. Chorioamnionitis was observed in 94.4% of births occurred between 21-24 weeks of pregnancy [4], while it was found in less than 5% of term births (over 37 weeks of pregnancy) [18]. Chronic chorioamnionitis occurs in 34% of premature births, of which in 39% with rupture of fetal membranes [19,20], and this form is especially common in late premature births in as many as 70% of proven cases [19,21]. This implies that its frequency is directly proportional to gestational age in premature births.

Histological analysis of the placenta, as the gold standard, can demonstrate the presence of chorioamnionitis in 33% of preterm births with preserved amniotic membranes and in as many as (approximately) 80% of preterm premature rupture of amniotic membranes (PPROM) [9, 22]. Nasef and coworkers [23] monitored the incidence of histological chorioamnionitis in relation to gestational age, and observed that it was found in 41% of births before 27 weeks, 15% of births between 28 and 36 weeks, and in only 2% of births after 37 weeks [24]. In the term births, histological chorioamnionitis was present in 20% of patients [25,26]. However, the marked decrease, regarding the incidence of histological chorioamnionitis in the term deliveries (from 2% to 20%), was registered because the histopathological analysis of placenta was performed only in those cases where there was a clinically justified suspicion of chorioamnionitis. Moreover, we are closer to the belief of the higher chorioamnionitis incidence in term deliveries (due to the lack of clinical manifestation) [27]. This could be an explanation for the partial overlap of two forms of chorioamnionitis - clinical and histological.

Chorioamnionitis is associated with intraamniotic infection. In as many as 72% of cases, the isolation of bacteria from amniotic fluid in preterm births was accompanied by histopathological confirmation of chorioamnionitis [9]. However, the detection of microorganisms in amniotic fluid is not enough to induce, but also to confirm diagnosis of chorioamnionitis [9]. In literature data, the percentage of noninfectious histological chorioamnionitis ranges from less than 30% [27] to more than 50% [28]. This could be an explanation for the partial overlap of histological and infectious form of chorioamnionitis.

Taking into consideration the clinical criteria, chorioamnionitis is recognized in 5-12% of term pregnancies [29,30] and in almost 20% of pregnancies with premature rupture of fetal membranes [14,31]. Among patients with clinical findings of chorioamnionitis, in 54% was detected intraamniotic inflammation with the presence of microorganisms, in 24% of patients presence of intraamniotic inflammation was not accompanied with presence of microorganisms, while 22% of women had no intraamniotic infection [29,32]. This could

explain the partial overlap of the two forms of chorioamnionitis - clinical and infectious.

HISTOLOGICAL CHORIOAMNIONITIS

The histopathological analysis represents the gold-standard diagnostic method for diagnosing chorioamnionitis [13]. Although with the greatest specificity, the sensitivity of this diagnostic procedure is quite low. This is evidenced by the weak association between histologically proven chorioamnionitis and the fetal inflammatory response syndrome (complications in fetuses caused by chorioamnionitis) [33]. Nevertheless, evaluation of histological chorioamnionitis represents the most secure diagnostic procedure, with the least possibility of false negative cases.

Acute histological chorioamnionitis is defined as an acute infiltration of granulocytes (neutrophils) of the choriodecidual space (maternal origin) and chorioamniotic membranes, chorionic villi, amniotic fluid and umbilical cord (fetal origin) [25]. By analyzing the origin of neutrophils (maternal or fetal), the exact origin of inflammation can be determined quite easily [4].

Neutrophils are usually not present in the chorioamniotic membranes and migrate from the decidua into fetal membrane in case of acute inflammation (neutrophils of maternal origin) [4,34]. However, they are present in the intervillous spaces of the placenta, but not in the chorionic plate [4,35]. The chemotactic gradient pulls them from the intervillous spaces into the chorioamnion membranes and the chorionic plate, which explains the maternal origin of neutrophils in acute chorioamnionitis (over 90%) [35].

Acute funisitis, vilitis and inflammation of the amniotic fluid are characterized by infiltration of neutrophils of fetal origin [36]. Previous studies showed a higher prevalence of interleukin 8 (IL8) as a chemotactic factor, as well as a higher expression of genes that increase the susceptibility to the inflammatory response in the umbilical vein wall than the artery [37]. The umbilical vein is the first vessel developing inflammatory changes, while umbilical arteritis represents a sign of advanced inflammation and carries a higher risk for neonatal complications [2, 26]. For that reason, in early detection of inflammation, the umbilical vein should be the first choice vessel for puncture and blood collection for laboratory analyzes of umbilical cord blood. The most serious form of funisitis is the migration of neutrophils into Warthon's jelly [4,37]. This form of inflammation is called necrotizing funisitis [12]. However, funisitis is present in about 60% of cases [14,38].

Histological analysis of the placenta with the proven chorioamnionitis, based on the localization of neutrophils, defines three stages of inflammation:

Acute histological chorioamnionitis 1 (AHCA 1)

AHCA 1 is characterized by inflammation of the decidua and chorioamniotic space. These are the first tissues affected by the inflammatory process, and neutrophils are of mother origin [25]. It is most often detected by analysis of placenta in preterm labor (after 37 weeks) and is considered to be triggered by an inflammatory response (usually aseptic), which represents the physiological mechanism of labor initiation [25,26]. In the literature, synonymous for AHCA 1 such as deciduitis with or without chorioamnionitis can be found.

Acute histological chorioamnionitis 2 (AHCA2)

AHCA 2 is characterized by inflammation of the amnion and / or chorion without umbilical cord inflammation, ie. by infiltration of neutrophils of mostly fetal origin. It is most common in births between the 32nd and 36th week of gestation, and represents the most common form of histological chorioamnionitis [2].

Acute histological chorioamnionitis 3 (AHCA 3)

AHCA 3 is an advanced inflammatory process that occurs in early preterm birth (<32 weeks) [2,25]. It is characterized by an intense fetal inflammatory response, and represents the final degree of extraplacental chorioamnion inflammation [39,40] along with neutrophilic infiltration of the umbilical cord. It is synonymous with necrotizing funisitis.

Histological chorioamnionitis of acute origin is more common and more severe in preterm, in relation to term births [41], and even three times more often in relation to clinical chorioamnionitis. It occurs in the term (AHCA 1) with a frequency of 7-85%, while the prevalence in preterm births (AHCA 2 and AHCA 3) is 4-63% [42]. The literature data more precisely showed the frequency of histological chorioamnionitis in 20% of term deliveries, and 38-50% of preterm deliveries [43].

Chronic histological chorioamnionitis is defined as chronic infiltration of the placenta (decidua, chorioamnion membranes, chorionic plates and chorionic villi) by lymphocytes, plasma cells and / or macrophages [19,44]. In the largest percentage of cases, the etiology of chronic chorioamnionitis remains unknown [45]. However, infectious agents and fetomateral immune response (by type of allograft rejection) are suggested as possible reasons [20,46-52].

The latter theory is supported by the following explanation - initiation of the mother's (host's) immune response to the father's antigens (expressed in the placenta, but also in the fetus). The main effectors of this immune response are cytotoxic lymphocytes (CD8 TLy) [20,45,53]. This condition is associated with the presence of fetal antibodies to leukocytes in maternal serum and syncytiotrophoblast [54].

In the strict sense of the term, chronic chorioamnionitis is characterized by infiltration of mononuclear cells in the chorioamnion membranes. Diffuse or uneven infiltration of maternal CD8 TLy from the decidua, primary to the levae zone of chorion where interaction of these cells with trophoblast cells occurs [20] consequently led to apoptosis of trophoblast cells. The migration of CD8 TLy was stimulated by the

chemotactic factors CXCL9, CXCL10 and CXCL11 from the chorioamnion membranes [55-64]. Under normal conditions, there is no inflammatory reaction in the choriodecidual junction, due to the expression of non-polymorphic HLA-G by trophoblasts [65-68] and the silenced T-cell chemokines gene in decidual cells [69].

The gradation of chronic histological chorioamnionitis is based on the degree of lymphocyte infiltration:

- grade 0 - without infiltration,
- grade 1 - over 2 foci of infiltration or uneven infiltration,
- grade 2 - diffuse infiltration [19].

Chronic chorioamnionitis in a broader sense includes chronic vilitis, as well as chronic deciduitis. Chronic vilitis is characterized by infiltration of CD8 TLy of maternal origin [53], as well as macrophages of fetal origin (Hofbauer cells) [70]. It is a unique inflammatory process in which inflammatory cells originating from two different participating hosts. The migration of CD8 TLy is stimulated by the chemotactic factors CXCL9, CXCL10 and CXCL11 from macrophages, endothelial cells and stromal cells [46]. Chronic deciduitis is diagnosed by the presence of lymphocytes and plasma cells in the decidua [71].

CHORIOAMNIONITIS	CHARACTERISTICS	DELIVERY	REFERENCE
Acute histological	Neutrophils infiltration	Treterm and term	Conti N. and al. [25]
AHCA 1	Decidua and chorionic space	Mostly term, ≥ 37 week	Conti N. and al. [25] Lee SM and al. [26]
AHCA 2	+ amnion and/or chorion	Mostly preterm, 32 -36 week	Torricelli M. and al [2]
AHCA 3	+ umbilical cord	Mostly preterm, ≤ 32 week	Torricelli M. and al [2] Conti N. and al. [25]
Chronic histological	Lymphocyte infiltration (CD8 TLy) with/without macrophages and plasma cells	preterm (34-70%) and term	Kim CJ and al. [19] Murphy HS [44]
In narrow sence	Chorioamniotic membranes	term/preterm	Kim CJ and al. [20]
In wider sense	+ decidua и chorionic villi	term/preterm	Reyes L and al. [53] Khong TY and al. [71]
Infectious	pathogens – bacteria, viruses, fungi	Term, ≥ 37 week (10%) Preterm, 32 -36 week (18%) Preterm, ≤ 32 week (39%)	Torricelli M. and al [2]
bacterial	Ureaplasma urealiticum (47%) Gram negative anaerobes (38.4%) Micoplasma hominis (30.4%) Bacteroides bivius (29.5%) Gardnerella vaginalis (24.5%) Streptococcus agalactiae (15%) Escherichia coli (8%)	preterm (39%) term (10%)	Torricelli M. and al [2] Czikk MJ and al [15] Redline RW and al [18] Hecht JL and al [103]
viral	Predominantly adeno viruses	preterm (41%)	Czikk MJ and al [15] Spiegel AM and al [104]
fungal	Candida sp.	term/preterm (<0.8%)	Stock SJ and al [105]
Clinical	Maternal hyperthermia (>37.8^{0}C) (95-100%) Maternal tachicardia (>100 beat per minute) (91.1%) Fetal tachicardia (>160 beat per minute) (66%) Maternal leucocytosis (>15000/mm^3) (33%) increased tenderness of the uterus (9%) unpleasant smell of amniotic fluid (3%)	preterm (20%) term (5-12%)	Tita AT and al [14] Romero R and al [29] Martinelli P and al [109] Burke C and al [113] Romero R and al [117]
Biochemical	Increase in IL6 and IL8 concentration	Preterm and term	Romero R and al [29] Burke C and al [113] Evers AC and al [114]

INFECTIOUS CHORIOAMNIONITIS

Positive bacterial cultures were found in 10% of placenta from term, 18% of placenta from preterm births (between 32-36. weeks of pregnancy), while the highest percentage (39%) of placenta with positive bacterial cultures was obtained from childbirth before the 32nd week of pregnancy [2]. Histologically confirmed chorioamnionitis is found in 72% of cases of preterm births in which microbiological invasion of amniotic fluid was detected by amniocentesis [9]. Interestingly, in 40-60% of cases, aseptic intraamniotic inflammation was found in verified histological chorioamnionitis [72-75].

Although the etiology of chorioamnionitis remains elusive, the role of microorganisms, especially bacteria, cannot be disputed. It is considered that the development of infectious (microbiological) chorioamnionitis is only one of the stages in the development of intraamniotic infection. The dilemma remains whether intraamniotic infection is the cause or consequence of chorioamnionitis [9]. Also, it is not entirely clear whether chorioamnionitis is a cause or a consequence of preterm premature rupture of membrane (PPROM) and premature birth [9,76].

However, the presence of microorganisms in amniotic fluid is not always the indicator of microorganisms in the placenta. Also, it does not mean the development of chorioamnionitis. There are a number of reasonable explanations for these claims: the critical concentration of microorganisms as inducers of preterm birth or chorioamnionitis in the intrauterine compartments cannot be defined [77-81]. Secondly, the enzymes of almost all microorganisms are not sufficient/enough, to solely cause chorioamnionitis and premature birth. Therefore, it is considered that the host immune system triggered by microorganisms (or their particles) is responsible for the occurrence of the above conditions [82-84].

An interesting fact is that acute histological chorioamnionitis was found in 37% of cases with a negative finding of microorganisms in amniotic fluid [85]. In that study, sample of amniotic fluid was collected by amniocentesis, just before delivery (up to 48 h). The transcervical approach was excluded because it increases the possibility of secondary contamination, as well as the number of false-positive results.

Bacteria are the most common cause of infectious chorioamnionitis. Bacterial DNA was detected in 50% of cases, and the microbiome ranged from vaginal to oral [86]. Regarding to gestational age, gram-positive bacteria were detected in 17% of placenta, while gram-negative bacteria were found in 10% of placenta in births before 32 weeks of age. In 11% of placentas, presence of gram positive, and in 8% gram negative bacteria were registered in childbirth between the 32nd and 36th week of pregnancy. In term, the placenta contained gram positive bacteria in 5%, and gram negative bacteria in 4% of cases [2].

Under normal conditions, the amniotic cavity is sterile [4]. The most common pathway for infection spreading is ascending from the lower genital tract. However, the mucus plug is an anatomical and functional barrier, which normally prevents infection spreading [87-93]. Furthermore, the invasion of bacteria into amniotic cavity is not exclusively dependent from membranes rupture. Fetal membranes have similar function, although they not represent an absolute barrier. There is no need for rupture of membranes for bacteria to be present in the amniotic cavity. In favor of that goes the finding of the initial infection of decidua in the supracervical region. Subsequently, bacteria multiply and pass through the chorioamnion membranes [94,95].

Bacteria are more often detected in amniotic fluid than in chorioamniotic membranes (100% and 33%, respectively) [96]. Moreover, it can be found in chorioamniotic membranes (probably as the initial stage of invasion), although in that case bacteria cannot be isolated from amniotic fluid [97]. It was not considered for the initiation of premature birth, except in the case of bacteria introduced into amniotic cavity [98]. In such a case, a strong inflammatory reaction is activated with an increase in the concentration of proinflammatory cytokines and chemokines [4].

Hematogenous dissemination of microorganisms is one of the pathway of spreading infection from primary focus to gravid uterus. The fact of more frequent intraamniotic infections and more frequent chorioamnionitis in women with periodontal diseases speaks in favor of this circumstance [99,100]. Microorganisms reach the intervillous spaces, invade chorionic villi and enter the fetal circulation [101]. Iatrogenic infection can be introduced into the amniotic cavity during diagnostic or therapeutic procedures, while the retrograde route of infection spreading from the abdominal cavity through the fallopian tubes cavity remains debatable [102].

The percentage of confirmed positive bacterial cultures is directly proportional to the severity of histological chorioamnionitis. Bacteria were isolated in 18% of AHCA 3 cases, 12% of AHCA 2 cases and only 4% of AHCA 1 cases [2]. Also, positive bacterial cultures in placentas was positive in 39% of preterm births and 10% of term deliveries [2].

The most commonly isolated bacteria in amniotic fluid are *Ureaplasma urealiticum* (47%), gram negative anaerobes (38.4%), *Mycoplasma hominis* (30.4%), *Bacteroides bivius* (29.5%), *Gardnerella vaginalis* (24.5%), and *Streptococcus agalactiae* (15%) [15]. Microbiological analysis of the placenta revealed *Ureaplasma urealiticum* (47%), *Gardnerella vaginalis* (26%), while *Escherichia coli* was present in about 8% of cases [18,103]. In 65% of cases, the isolated flora was polymicrobial [14]. Isolation of the virus from amniotic fluid is less frequent than the isolation of bacteria, although as the most dominant among viral represent adenoviral infection of the placenta (41% of premature births and 75% of histologically proven chorioamnionitis) [15,104]. Fungal infections were found to complicate less than 0.8% of pregnancies [105].

The concept of "TRIPLE I". In the light of etiological considerations, chorioamnionitis was observed as heterogeneous entity. Several American health associations have suggested that the term chorioamnionitis could be replaced by the term "triple I" - infection, inflammation and both [8,12]. Although this term can be used in clinical infectious chorioamnionitis, and confirmed by bacteriological examinations, it still remains reserved for the histological form of chorioamnionitis [8,12].

"Triple I" encompasses the two most important pathophysiological processes; infection - which is found in a significant percentage of chorioamnionitis, and inflammation that can be aseptic or triggered by microbiological invasion. It also refers to any of these two terms isolated: infection (proven microbiological agents, their particles or DNA) of placental tissue, fetal membranes, amniotic fluid and umbilical cord, without consequent inflammation [9], or aseptic inflammation without proven microorganisms [27].

"Triple I" is interpreted in terms of clinical manifestations of chorioamnionitis, and confirmed by microbiological, histopathological and laboratory analyzes [10,106,107]. However, a recent Cochrane review concluded that the quality of the evidence in favor of the broader application of the “triple I” concept is poor [8,108].

CLINICAL CHORIOAMNIONITIS

The prevalence of clinical chorioamnionitis ranges from 5-12% of term pregnancies, and 20% of preterm pregnancies with premature rupture of the fetal membranes [14,29,109]. Microorganisms in amniotic fluid are present in 61% of patients with clinical chorioamnionitis at the term gestations, but the diagnosis is rarely confirmed by microbiological tests [29,110]. However, in about 24% of cases of clinical chorioamnionitis, no microorganisms were detected, despite presence of intraamniotic inflammation [29]. Overlap of clinical and histological chorioamnionitis was found in 51-62% of cases [111].

Clinical chorioamnionitis is an entity characterized by maternal temperature of 38°C or higher and, at least two of the following signs:

- maternal tachycardia (> 100 beats per minute);
- fetal tachycardia (≥ 160 beats per minute);
- maternal leukocytosis (≥ 15000 leukocytes / mm^3);
- increased tenderness of the uterus;
- unpleasant smell of amniotic fluid [12,112].

Maternal hyperthermia almost always exists in clinical chorioamnionitis (95-100%) and represents the most important clinical sign. However, it is an accurate indicator of a proven infection in the microbiological form in only 30% of cases [113,114]. This finding indicates that not every hyperthermia is of infectious etiology, although the perinatologist can start the treatment with antibiotics in presence of hyperthermia [113].

The reason for the hyperthermia during delivery may be a consequence of the applied epidural anesthesia. In these circumstances, there is an increase in vaginal temperature at a rate of 10°C every 7 hours [113,115]. The exact mechanism of epidural anesthesia on hyperthermia occurrence remains unknown, but it is assumed that sympathetic blockade of thermoregulatory processes underlied it [14]. However, there is no reliable way to distinguish the hyperthermia of infectious etiology and hyperthermia caused by epidural anesthesia [113,116].

Maternal tachycardia (present in 91.1% of patients with chorioamnionitis) may not always be an indicator for pathological condition [117]. It may occure due to significant maternal hemodynamic needs, increased heart rate and cardiac output [113,118]. Also the use of medications (sympathomimetics), as well as psychosomatic stimuli that activate the sympathetic nervous system may be the cause of maternal tachycardia.

Fetal tachycardia occurs in 66% of chorioamnionitis [113,117]. It is a consequence of increased fetal metabolism in conditions of maternal hyperthermia, while it also occurs isolated in presence of fetal hypoxia due to compensatory stimulation of the sympathetic nervous system [113,119]. Diagnostic accuracy of fetal tachycardia in chorioamnionitis is partial because it is not always a reflection of infectious etiology (as evidenced by the previous statements).

Leukocytosis is considered to be a number of leukocytes in the mother's blood higher than 15000 leukocytes / mm^3 and represents a non-specific biochemical indicator. It can be registered during delivery, even without evidence of infection. It occurs in 33% of cases of clinical and histologically proven chorioamnionitis [113,117]. Tita and Andrews [14] found a higher frequency of leukocytosis in clinical chorioamnionitis (70-90%), but they defined leucocytosis as higher then 12000 of leukocytes in mm^3 of blood.

Increased uterine tenderness occurs in 9% of chorioamnionitis, but it is accurate indicator in predicting clinical chorioamnionitis in 48.9% of cases [113,117]. It occurs due to prostaglandins release as well as secretion of bacterial exo- and endotoxins, and thus increased uterine contractility [113,120]. Moreover, decreased uterine perfusion and consequent muscle hypoxia may cause increased uterine tenderness as well as blood in the decidua [113,120].

Unpleasant malodour of amniotic fluid is found only in 3% of chorioamnionitis, while its diagnostic accuracy was 46.3%. It is most commonly associated with intraamniotic bacterial infection [113,117].

Signs and symptoms of clinical chorioamnionitis have low sensitivity and specificity, poor prediction, are not reliable and usually appear late. They may be absent, despite proven histological confirmation of chorioamnionitis, but also present in cases of histologically unconfirmed chorioamnionitis. Nevertheless, they represent an empirical guideline

for the dilemma in the application of therapy during pregnancy.

BIOCHEMICAL CHORIOAMNIONITIS

This form of chorioamnionitis is the most controversial and debatable. Literature data do not provide enough information about biochemical chorioamnionitis. It is difficult to even define this term. Simply, we could state that biochemical chorioamnionitis is found with more or less share in all the previously mentioned forms.

Complex biochemical processes represent the fundamental links in all pathophysiological processes, as well as in mechanisms underlie the chorioamnionitis. Beside various processes, apoptosis is one of the basic mechanism affecting and damaging the trophoblast cells [19,20]. Chemotaxis, as a basis for the attraction of inflammatory cells, is also mediated by complex biochemical processes. IL8 acts as a chemotactic factor for neutrophil infiltration in acute chorioamnionitis [4,37]. Chemokines such as CXCL9, CXCL10 and CXCL11 released from macrophages, endothelial cells and stromal cells attract cytotoxic CD8 TLy in complex immune and pathophysiological processes of development of chronic chorioamnionitis [19,46].

Determining the above-mentioned immune components in the blood of the mother, umbilical cord or fetal blood can be of help in a better understanding of chorioamnionitis. The specific analysis of biological tissues (samples of placental tissue, biopsies of decidua or umbilical cord) using sophisticated biochemical procedures represents very interesting and scientifically based procedures. Cervical smear for fibronectin is a decades-long test in assessing the risk of premature birth [9,121-124], followed by determination of salivary estradiol [125,126] or corticotropic hormones. Standard inflammatory markers such as fibrinogen, procalcitonin, and C-reactive protein are nonspecific when observed isolated, but they were commonly interpreted along with leukocytosis [9].

Traditional determination of proinflammatory cytokines (TNF, IL1 and IL6) in blood or amniotic fluid, as well as antiinflammatory cytokines (TGF, IL10), chemokines, their receptors and receptor agonists is less and less specific for chorioamnionitis [9]. Currently, IL8 is the most reliable diagnostic marker for the pathohistological confirmation of acute chorioamnionitis [113,114]. However, determination of the IL6 concentration in amniotic fluid is used to assess the extent of the inflammatory response [29]. The mean value of IL6 concentration in clinical, microbiologically confirmed chorioamnionitis is 14 ng / ml [29].

There is an urgent need for further investigations in order to detect a specific biochemical marker, while known biomarkers have not given the expected results so far [9]. Probablly, the future diagnosis will rely on determination of so-called "alarmins", a more sensitive and specific markers of cell damage, such as IL33 [29,127]. The rationale for such considerations are apoptotic processes of the placenta trophoblast cells that occur during chorioamnionitis. Studies concerning this theme are ongoing.

CONCLUSION

Taking into consideration the above mentioned, it could be abslutely argumented, that chorioamnionitis represents a great enigma for perinatologists. Although significant progress has been made in understanding pathophysiological events, it turns out that the key "links" of this complex process are often missing or unclear. In support of that, there are constant dilemmas: is intraamniotic infection a cause or a consequence of chorioamnionitis? Even more, it is not entirely clear whether chorioamnionitis is a cause or a consequence of PPROM and premature birth?

The diversity of the chorioamnionitis prevalence is a consequence of insufficiently defined criteria, especially when it comes to clinical chorioamnionitis. However, there is less of a problem regarding the clinical signs (hyperthermia) than clinical symptoms (uterine tenderness). Generally, the sensitivity and specificity remains extremely low, so the diagnosing of chorioamnionitis in this way is certainly questionable.

A series of diagnostic procedures performed in order to confirm of chorioamnionitis did not give the expected results. Standard laboratory inflammatory markers are overcomed, and interpreted only in the context of clinical manifestations. Diagnostic procedures such as amniocentesis are more sensitive, but they are invasive and not routinely performed in purpose of diagnosing inflammation of amniotic fluid or placental tissue.

Definitive confirmation of chorioamnionitis is made by histopathological analysis which represents the gold standard. However, the incidence of histological chorioamnionitis is three times higher than clinical, which is an additional aggravating circumstance. Although, the histopathological diagnosis of chorioamnionitis remains the most reliable one, it can be performed only after delivery i.e. retrogradely. Nevertheless, after delivery, there is no further effect of the medications on the course of pregnancy, or on the potential complications of the newborn.

Further investigations regarding chorioamnionitis, could provide a deeper understanding of pathophysiological events and detection of specific and sensitive biomarkers. The detection of "alarmins", specific cytokines indicating cell damage, should also be taken into consideration. Furthermore, special attention should be paid into initial factors, the initiators of the aseptic inflammatory reaction, which is both clinical and scientific challenge.

All of the abovementioned should provide a more effective perinatological monitoring, prevent potential neonatological complications, decrease the time between diagnosis and therapeutic treatment, but also provide more precise evidence based facts and taxonomy.

CONTRIBUTORS

All authors contributed to and have approved the final manuscript.

CONFLICT OF INTEREST

The authors report no conflicts relevant to this paper.

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MEMANTINE THERAPY AS AN AUGMENTATION IN TREATMENT-RESISTANT DEPRESSION: A CASE REPORT

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ABSTRACT

Evidence suggests that treatment-resistant depression (TRD), defined as the occurrence of an insufficient clinical response after adequate antidepressant therapy among patients diagnosed with major depression, is one of the most important public health problems and is associated with significant disability and psychosocial impairment. Here we present a case of depression resistance to the treatment of a 22-year-old man and the effects of Memantine augmentation. The first symptoms of depression appeared in January 2019, and pharmacotherapeutic treatment began in March of the same year. He was in a bad mood, sad, hopeless, tense, lacked energy, lost interest, and decreased libido. During his two-year pharmacotherapeutic therapy, he took SSRIs, SNRIs, TeCAs, SARIs, atypical antipsychotics with antidepressant effects, such as aripiprazole and cariprazine. Since the patient still did not have adequate therapeutic response and new drugs were available, the choice fell on Memantine after researching several databases and collecting the data listed at the beginning of the article. The initial dose was 5 mg daily and was gradually increased by 5 mg each week to a maximum of 20 mg. After 2 weeks at a stable dose, the patient reported feeling a little better. Significant improvement in symptoms was observed in the MADRS score, which was then applied to assess the effects of augmentation. After four weeks of therapy, he entered remission which is still ongoing.

Keywords: *Major depressive disorder, treatment-resistant depression, memantine, pharmacotherapy.*

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INTRODUCTION

Treatment-resistant depression (TRD) is broadly defined as the occurrence of an insufficient clinical response following adequate antidepressant therapy (in terms of dosage, duration, and compliance) among patients diagnosed with major depression (1).

According to the European Staging Method TRD is defined as:

A. Nonresponder to TCA, SSRI, MAOI, SNRI, ECT, other anti-depressants. No response to one adequate antidepressant trial in 6-8 weeks.
B. Resistance to 2 or more adequate antidepressant trials. Duration of the trial(s): TRD1:12-16 weeks; TRD2:18-24 weeks; TRD3:24-32 weeks; TRD4: 30-40 weeks; TRD5: 36 weeks-1year.
C. Chronic resistant depression - Resistance to several antidepressant trials, including augmentation strategy in the duration of the trial at least 12 months (2).

Evidence indicates that TRD is one of the most important public health problems and it is associated with significant disability and psychosocial impairment (3). Clinical practice has shown that individual treatment of almost every patient is necessary due to heterogeneity that exists in the psychiatric population. Various clinical studies have examined the effects of NMDA antagonists in mood disorders. Evidence indicates an alteration in the glutamatergic system in major depressive disorders as well as in treatment-resistant depression. Besides the well-known monoamine hypothesis, research suggests non-competitive modern affinity N-methyl-D-aspartate receptor antagonists (NDMA) in treatment resistance depression, due to changes in NMDA level in this patient (4).

Memantine is an NMDA receptor antagonist, indicated for the treatment of Alzheimer's disease. The fundamental role of these receptors is to bind glutamate: the main excitatory neurotransmitter in the brain believed to play a crucial role in neuronal plasticity and learning mechanisms. The relationship between depression and the NMDA pathway is reinforced by the fact that people with MDD (major depressive disorder) also demonstrate higher glutamate levels in the brain and blood(5). Memantine significantly slows neurodegenerative processes and it is effective in treating symptoms of dementia, shows good toleration by patients, in combination with various psychiatric medications offers great potential in treatment. Studies have shown that the effects of the glutamatergic system include the delayed, indirect effects of many antidepressants as well as the antidepressant effects of N-methyl-d-aspartate (NMDA) receptor antagonists in basic and clinical models of depression. (6-8)

In this article, we present the case of a 22-year-old man who has treatment-resistant depression. This case may help to enrich our understanding effects of Memantine augmentation in this condition.

CASE REPORT

The patient had the first episode of depressive symptoms in January 2019. He was in a bad mood, sad, hopeless, tense, lacked energy, lost interest, and decreased libido. At the beginning of March of the same year, pharmacotherapeutic treatment began. In the first episode from March 6th, 2019 the patient first took Escitalopram 10mg daily and Alprazolam 0,75mg daily. Due to poor tolerability and worsening of symptoms in terms of lethargy, lack of motivation, loss of emotions, and withdrawal from social communications, the drug was replaced. He started taking Venlafaxine from an initial dose of 75 mg daily, which was then increased to 150 mg daily. A few days later, the symptoms of depression became even more pronounced, he became very anxious due to the loss of all positive and negative emotions, and at the next follow-up visit, which was on April 3rd. 2019, the dose of Venlafaxine was adjusted to 75 mg daily and a new drug Olanzapine has introduced at the dose of 5mg which the patient did not tolerate well and which were quickly excluded from therapy. As a result, at the beginning of April 2019, he was entering remission, at that time he was only taking Venlafaxine in a dose of 75 mg daily. The remission lasted until July 2019. Then the symptoms worsen and the current depressive episode begins. Among the problems, he mentioned a depressed feeling, loss of interest in activities that were a pleasure, a feeling of emptiness, loss of energy ... The patient has been prescribed Maprotiline for the first time in a dose of 25 mg three times a day from July 20th, 2019. At the next follow-up, the patient did not report improvement in discomfort, and either a combination of the two antidepressants or augmentation was considered. We decided to increase the dose of Maprotiline to 100 mg/day from September 3rd, 2019, and introduce the drug Trazodone in an initial dose of 50 mg daily for the first 3 days, and then 100 mg daily. The patient appears to be therapeutically resistant and at the next follow-up, on September 17th, 2019, since he did not report any improvement, this therapy was excluded and Escitalopram 10 mg daily and Aripiprazole 5 mg daily were introduced, which he took regularly until November 22nd, 2019, when started treatment with Duloxetine at a dose of 30 mg and later 60mg daily. He did not come until June 5th, 2020, when he stated that the condition was much worse. Considering that patient in this episode still did not have an adequate therapeutic response and new medication (Cariprazine) which can be used for therapy of augmentation was available, Cariprazine was prescribed in the therapy.

At that moment, since he is a very young patient with severe problems and significant obstruction in his functioning, more intensive monitoring began.

At that moment, he was in a bad mood, slowed down, he had no wellness or energy, he was feeling empty, he lost interest in friends, family and all the things that used to be a pleasure, his concentration was bad, he had a drop in libido...

The diagnosis was confirmed according to the DSM-V criterion and confirmed according to the SCID-5 scale. The

diagnosis of treatment-resistant depression was made according to the criteria of therapy resistance. The patient did not have adequate response and improvement to more than two therapeutic protocols that were used long enough and at a sufficiently high dose (Table 1) (2).

Medical history was indicated that the patient is not treated for other significant medical conditions other than depression.

Evaluation of the severity of the disease with the Montgomery-Åsberg Depression Rating Scale (MADRS) has begun. The MADRS depression scale was performed on the first day of treatment with Cariprazine from July 30 and repeated until September 9th (Table 2). No significant improvement in symptoms was observed, so he left this therapy on his own.

At that moment, the authors of this article, considered which alternative protocol can be used in this case. After researching several databases and obtaining the data listed at the beginning of the article, the choice fell on Memantine. Memantine was prescribed together with Duloxetine. The patient was informed about the mechanism of action of memantine, its potential side effects, and the off-label use of the drug before starting it. The patient consented to add-on treatment with Memantine. In the beginning, the dose was 5mg, and every week it was increased by 5 mg to a maximum of 20 mg. Dosing was increased according to instructions used in previous trials (9). After 2 weeks on a dose of 20 mg, the patient reported feeling a little better and the psychiatric evaluation of the MADRS was 16 points. After another week, the total score on MADRS decreased to 13 points, and after another week to 11 points. When he came for an appointment after another month of treatment, the MDRS was 9 points. Table 3 The patient reported that he had not felt so well in a long time.

The patient reported that he felt better. His mood corresponded to the situation. He was not depressed. He had much more energy. His motivation and initiative were like in the period before the disease, his libido returned. Also, its functioning has improved. The patient started to achieve success at the faculty again and fulfilled all the planned obligations. Treatment did not change since then. From the beginning of the treatment, the patient tolerated the treatment well and no side effects were observed. The patient is still in stable remission.

DISCUSSION

In this case study, we reported a case of a 22-year-old man with depressive symptoms according to DSM-V, who had a significant improvement in symptoms after Memantine augmentation.

The first results on the efficacy of NMDA antagonists in treatment are shown by Berman et al. They found that a single dose of the high-affinity NMDA receptor antagonist ketamine resulted in a rapid antidepressant effect in patients with major depression. Unfortunately, possible lack of ketamine treatment is psychotomimetic effects which preclude its use as a chronic antidepressant (10)

Réus et al. (2009) suggest that acutely administration of Memantine (20mg/kg), significantly increased BDNF protein levels in the rat hippocampus as well as inhibition of the reuptake of serotonin and dopamine into mouse forebrain synaptosomes and effects on cholinergic signaling via muscarinic and sigma receptor in the brain tissue (11). Antidepressant-like effects of Memantine could be a consequence of interactions with several receptor systems involved in the modulation of behavioral and molecular actions of antidepressants. Moreover, Onogi et all (2009) showed increased locomotor activity after Memantine administration to mice. They also suggested that the D2 receptors of the nucleus accumbens could be involved in this effect. The administration of Memantine may inhibit MAO activity in the human brain, as well as have effects through NMDA receptor antagonism (12). The result of our case study is consistent with 12 weeks of open-label treatment study of 8 subjects with major depression conducted by Ferguson et al, which showed that Memantine may provide rapid and potent relief of depressive symptoms in patients with MDD. They administrated Memantine in dosage from 20 mg/day to 40mg/day and showed that therapy was well tolerated at the highest dosage 40 mg/d which is twice the dosage commonly prescribed for patients with Alzheimer's disease. The study limitations were lack of placebo and active controls were not used, and the possibility that results stem from a placebo response cannot be ruled out (13).

In contrast to previous research placebo-controlled trial of a selective NMDA antagonist in the treatment of major depressive disorder failed to show that Memantine has antidepressant effects in patients with major depression. Potential limitations of this study are the small number of subjects and low doses of Memantine (14).

CONCLUSION

Memantine therapy as an augmentation in TRD is successful in some cases. Considering that this drug is well tolerated and that the side effects are milder and less frequent than with some other augmentation drugs, it leaves room for further research in this field and can lead to progress in one of the most difficult fields of everyday psychiatric practice.

Table 1.

Medication	Dose (mg/ day)	Start / end time	Dose adjustment	Date/ time of dose adjustment
Escitatopram	10	06. March 2019/ 28. March 2019	NO	
Alprazolam	0,75	06. March 2019/ 28. March 2019	NO	
Venlafaxine	75 -150	29. March 2019/ June 2019	YES	Start dose 75 mg 29.03. - 04.04.2019 05.04-08.04. 150mg 08.04 - 06.2019. 75mg
Maprotiline	75 - 100	June2019/ September .2019	YES	06.2019-03.09.2019 - 75mg 04.09- 17.09. 10mg
Trazodon	100	03. September 2019	YES	03.09-10.09. 50mg
Escitaloprame	10	17. September 2019 / 22. November 2019	YES	17.09 - 24.09 5 mg, and than 10mg
Aripiprazole	5	17. September 2019 / 22. November 2019	NO	
Duloxetine	60	22. September 2019 -	YES	22.09-29.09 - 30mg, and than 60mg
Cariprazine	1.5-4.5	05. June 2020 - 09. September 2020	YES	05.06-19.06. 1.5mg; 20.06.-20.07. 3mg; 21.07-09.09. 4,5mg

Table 2.

Effects of Cariprazine therapy	
Visit date	MADRS score
30. July 2020	31
12.August 2020	31
19. August 2020	31
26. August 2020	29
09. September 2020	30

Table 3.

Effects of Memantine therapy	
Visit date	MADRS score
23. September 2020	16
30. September 2020	13
07. October 2020	11
04. November 2020	9

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IATROGENIC PNEUMOPERICARDIUM IN A MALE FULL-TERM NEWBORN WITH SPONTANEOUS PNEUMOTHORAX

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ABSTRACT

Neonatal pneumopericardium, a collection of air in the pericardial sac, is less common form of air leak syndrome, but unfortunately with high mortality rate. We report a rare case of male full-term newborn who soon after birth presented with respiratory distress. Chest radiograph showed spontaneous bilateral pneumothorax after which a chest drain was placed between anterior and midaxillary line in the 5th right intercostal space. The infant soon presented with tachypnea, dyspnea, muffled heart sounds, acidosis indicating cardiorespiratory worsening. On chest radiograph ''Halo'' sign appeared indicating pneumopericardium. We believe that spontaneous reposition of a chest drain damaged the pericardial sac which combined with ventilation mechanism (''Macklin effect'') most likely led to pneumopericardium. After partial chest drain extraction the infant showed signs of improvement, but had to be closely monitored due to risk of tension pneumopericardium. Careful thoracal drain placement and fixation is crucial to prevent iatrogenic pneumopericardium, which can lead to deadly tension pneumopericardium.

Keywords: *Newborn, iatrogenic disease, pneumopericardium, chest tube.*

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INTRODUCTION

Neonatal pneumopericardium (PPC) is a pathological collection of air in the pericardial sac. It is less common form of air leak syndrome, but unfortunately with high mortality rate. The majority of pneumopericardium now occurs secondary to the more common air leaks, e.g. pneumothorax, pneumomediastinum, pulmonary interstitial emphysema and is usually presented as a complication of mechanical ventilation, mainly in preterm newborns. Neonatal air leak syndrome is usually accompanied by surfactant deficiency lung disease, mechanical ventilation, active resuscitation or other chest trauma. Isolated PPC in nonventilated newborns is a very rare occurrence. Due to some changes in the medical practice that have happened in the last few decades, such as the increasing use of antenatal steroids, exogenous surfactant therapy, as well as gentler ventilation techniques, the incidence of all air leak syndromes has decreased significantly. Some patients are clinically asymptomatic but there are those with various symptoms such as, chest pain, palpitations, hypotension, respiratory distress or different electrocardiographic findings. Early diagnosis is very important because there is risk of cardiorespiratory instability and a potentially life-threatening condition – tension pneumopericardium. We report a rare case of iatrogenic PPC after installment of a chest drain in a nonventilated male full-term newborn with bilateral apical pneumothorax. (1-7)

CASE PRESENTATION

A term male infant, weighing 2700 grams at birth, was born at 39 weeks to a healthy mother (blood type O, Rh positive) delivered spontaneously by vaginal route. Pregnancy was well monitored and unremarkable. The infant cried immediately after birth and did not require any interventions. He had a birth weight of 2700 grams, a length of 52 cm and a head circumference of 34 cm. Apgar scores were 9 and 9 at 1th and 5th minutes, and infant was transferred to the postnatal unit with his mother for regular postnatal care. During the third hour of age, his skin started to become diffusely livid with evidence of respiratory distress, tachypnea (respiratory rate = 63/min) and heart rate 136/min. He was transferred to the neonatal intensive care unit (NICU) for investigation and further management. On admission, his vital signs showed a temperature of 36.6 °C, heart rate of 101 per minute and respiratory rate of 68 per minute. His blood pressure was 77/50 mmHg and his weight reduced to 2680 grams. Capillary blood gas analyzed on a Gem Premier 3000 gas analyzer showed pH 7.05; PaCO2 13.1 kPa; PaO2 2,4 kPa; bicarbonate 27.1 mmol/l; base excess of −3,4 and SaO2 84%. Nasogastric tube was placed. The chest examination showed chest retraction as compared with abdominal retraction during inhalation – 2 points; retraction of the lower intercostal muscles – 1 point; xiphoid retraction – 0 points; flaring of the nares with inhalation – 1 point; grunting on exhalation – 1 point, suggesting Silverman score = 5 points which indicates moderate respiratory distress. In view of his respiratory distress, the infant remained in the incubator and hood with 40% fraction of inspired oxygen (FiO2) was applied and 20 mg of Theophylline (Aminophylline®) given 5mg/12hours. A chest radiograph revealed bilateral apical pneumothorax in Figure 1. A chest drain was placed between anterior and midaxillary line in the 5th right intercostal space. The procedure was done in aseptic conditions after the area was cleaned with 0.015% chlorhexidine and spontaneously dried for half a minute. A dose of 0.3ml/kg of 1% lignocaine was subcutaneously applied. An incision was made using a sterile scalpel above the inferior rib. An incision place was carefully pinpointed to avoid a well-known location of important neurovascular structures right below the superior rib. The chest drain was fixated using two sterile surgical stripes. The secondary capillary blood gas analysis showed pH 7.10; PaCO2 10.4 kPa; PaO2 3,4 kPa; bicarbonate 24.2 mmol/l; base excess of -5,5 and SaO2 was 89%. The following day radiological signs of bilateral partial pneumothorax showed mild signs of resolution. Blood gas analysis on showed pH 7.35; PaCO2 5.5 kPa; PaO2 3,8 kPa; bicarbonate 22.6 mmol/l; base excess of -3 and SaO2 was 94%. The next morning the infant had tachypnea and dyspnea with SaO2 89%. Cardiovascular examination showed muffled heart sounds with no audible murmur and heart rate 123 per minute. Chest X-ray showed complete resolution of pneumothorax however classical 'Halo' sign appeared, (a collection of air surrounding the heart in the pericardial sac) indicating existence of pneumopericardium, which can be seen in Figure 2. The infant became acidotic and SaO2 dropped to 83%. Due to inadequate fixation of the chest drain, it spontaneously moved and may have damaged the pericardial sac. Along with the already existing pneumothorax, probably a combination of ventilation mechanism and trauma resulted in air leak into the pericardial sac. The chest drain was therefore repositioned (extracted by 3 centimeters) and due to possible progression to life threatening cardiac tamponade the infant had to be closely observed with cardiorespiratory monitoring and series of chest radiographs. Over the course of next 24 hours the clinical and radiographical signs of pneumopericardium resolved. Respiratory rate was 42 per minute and heart rate was 115 per minute. SaO2 was 96%. Thoracal drain was then removed and latter subsequent chest radiographs showed no recurrence of either pneumothorax or pneumopericardium (Figure 3). The infant remained in incubator under diffuse oxygen therapy with FiO2 40% and in the following 48 hours his oxygen requirement was weaned gradually to room air and was discontinued the next day. He was discharged on 8th day of his life. His regular follow-ups showed he was thriving well.

Figure 1. A chest radiograph revealed bilateral partial pneumothorax

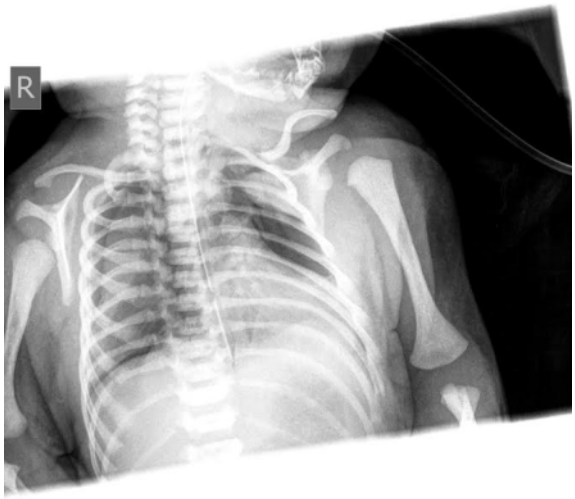


Figure 2. "Halo" sign of pneumopericardium on chest radiograph

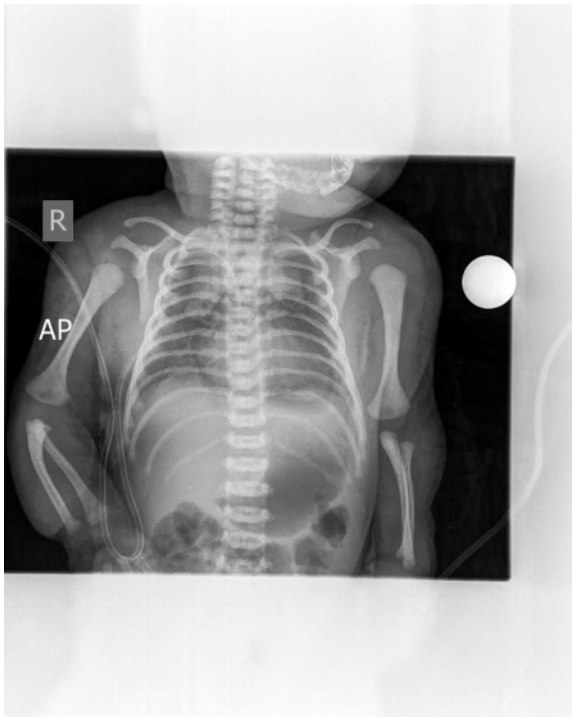
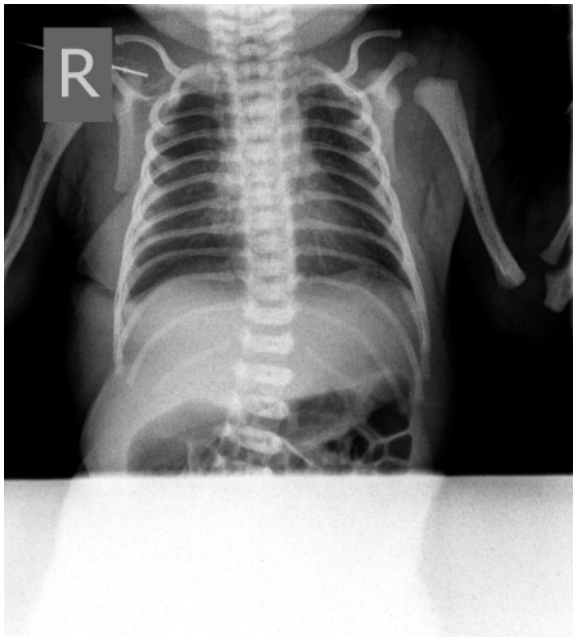


Figure 3. A chest radiograph after resolution of pneumopericardium and pneumothorax



DISCUSSION

In the year 1844, Bricheteau was the first to describe pneumopericardium, an abnormal presence of air in the pericardial cavity. PPCs occurrence can be divided into spontaneous or more often associated with positive pressure ventilation. In neonates, it can also be isolated or combined with other air leak syndromes, e.x. pneumothorax, pneumomediastinum, pulmonary interstitial emphysema. (3) Clinically, PPC can either be presented as non-tension or tension pneumopericardium, which is far more dangerous because it possibly leads to cardiac tamponade and deadly circulatory failure. (1) Spontaneous pneumothorax and PPC are rarely presented together in a term infant without mechanical ventilation, as in our case. (3) The exact pathophysiology of neonatal pneumopericardium is still unclear, however one of the possible mechanism thought to be responsible for pneumopericardium is the ''Macklin effect''. Alveolar rupture is the result of increased pressure gradient between the intraalveolar and the interstitial space. It is followed by air leakage into the pulmonary interstitium. Due to its connection with the peribronchial and pulmonary perivascular sheaths, air flows to the hilum of the lung and then to the mediastinum. From there on, air tracks near the area of pulmonary veins, at the place lacking collagenous tissue also described as ''anatomical area of weakness''. It flows into the pericardial sac resulting in pneumopericardium. (5) It is possible that if connection of pericardium is made with the bronchial tree air can also leek into its cavity. (4) The infant, in our case, cried immediately after birth and did not require any interventions. Pneumothorax that soon occurred is thought to

be spontaneous, and etiology of PPC is considered multifactorial. Combined ventilation mechanism (''Macklin effect'') with inadequate fixation of the chest drain that resulted in its deeper placement and possible tear of pericardium, most likely led to PPC. We believe this was the cause of PPC because after repositioning of the chest drain clinical and radiographical signs of the PPC disappeared in the following days. Clinical presentation of PPC can be asymptomatic or presented by various signs and symptoms, however none of them are specific. Patients could potentially have chest pain, dyspnea, tachypnea, palpitations, muffled heart sounds or different ECG findings. Some of them that are usually described are reduced ECG voltages, ST segment depression/elevation or T wave changes. (6) After initial clinical findings, a conformation of the diagnosis of pneumopericardium should be done using chest radiograph, which can be supported by computed tomography or echocardiography, however chest X-ray is considered a standard diagnostic method. The classical radiographical finding in PPC is a ''Halo sign'', recognized by air translucency outlining the heart and separating it from the lung fields by a strip of pericardium. (2,8) In our case chest radiographs showed iatrogenic PPC with spontaneous bilateral apical pneumothorax. Pneumomediastinum can be considered an important differential diagnosis. On posteroanterior chest radiograph a ''Continuous diaphragm sign'' can be seen in both pneumomediastinum and pneumopericardium as a translucency above the diaphragm. Although there are some radiographical similarities between there two pathologies, unlike PPC radiographic signs of pneumomediastinum show that air can stretch into the superior mediastinum, above the great vessels and around soft tissues of the neck. It also, unlike PPC, isn't limited by the pericardial layers and gives a radiographical presence of radiotransparent retrosternal triangle. (8-10) Ultrasound can also be used to differentiate PPC and pneumomediastinum. The ultrasound view of subxiphoid window in pneumomediastinum is transparent with possible visualization of the heart. Ultrasonography of pneumopericardium shows presence of gas in subxiphoid window and inability to completely visualize the heart. Another sign that can be seen in PPC are diffuse A lines on various ultrasound cardiac views. If differential diagnosis is still uncertain, chest computed tomography (CT), as a gold standard, should be considered as the tool to confirm the right diagnosis. Chest CT scan of PPC usually shows parietal pericardium visible as a thin line and a heart surrounded by air-density. (11, 12) Studies show that prenatal corticosteroid application and postnatal surfactant therapy lower the risk of neonatal hyaline membrane disease and therefore air leak syndrome as well. Pneumopericardium in neonates is treated conservatively, unless a patient presents with lowering of arterial blood pressure, muffled heart sounds or rise of jugular venous pressure (Beck's triad), which indicate life threatening cardiac tamponade. A little more than a third of simple pneumopericardium cases can progress to tension pneumopericardium, whose mortality rises up to 57%. Fortunately, the PPC in infant in our case did not progress to tension pneumopericardium. PPC therefore requires a close monitoring of oxygen saturation, heart rate, blood pressure and radiographical changes. (12-14) Should signs of cardiac tamponade (Beck's triad) and cardiovascular collapse occur, patient should be immediately treated, usually by needle aspiration. Subxiphoid window is a recommended location and the procedure should be followed by antibiotics to prevent infection. Chest drain could also be used to evacuate excess air from the pericardial sac, but the most important measure to prevent PPC when applicating chest tube is careful placement and good fixation. In our case needle aspiration was not needed but since inadequate chest drain position took part in occurrence of PPC, it is very important to highlight this and for doctors to take into consideration in their future practice. (13, 15, 16) It is believed that a''Nitrogen washout technique'', also known as oxygen therapy, could be a potentially beneficial treatment. Nevertheless, its use is limited because if it's used in premature infants born earlier than 32 weeks of gestation, there is a likelihood of it being associated with hyperoxia induced retinopathy of prematurity. Clark et al state that high levels of oxygen can also in newborns induce production of free radicals. This is because antioxidative protection in a neonatal period is not fully developed and this can lead to oxidative stress in many organs. In conclusion, there are different opinions among authors regarding the use of this technique. (2, 17, 18) Our patient improved after repositioning of right-sided thoracal drain while remaining in an incubator under diffuse oxygen therapy with FiO2 40%. The pneumopericardium resolved spontaneously without invasive treatment. Subsequent follow-ups showed his well improvement and good later development.

CONCLUSION

Iatrogenic pneumopericardium should be considered as a possible complication during invasive treatment, such as placement of a chest drain. This means that early diagnosis and close monitoring are crucial because it can lead to possibly deadly tension pneumopericardium, in which cases doctors should always be prepared to take immediate action. (16) Our patient has improved well with conservative treatment indicating that conservative approach is a reasonable choice in some clinical cases. Careful thoracal drain placement and fixation is a crucial action to prevent iatrogenic pneumopericardium. We believe that this case of iatrogenic pneumopericardium can help doctors to a great extent in their future practice, to mainly avoid this unnecessary complication.

CONFLICTS OF INTEREST

All authors read and approved to submit the manuscript and there is no conflict of interest. We don't have any sources of financial assistance for this research.

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