NASTAVNO-NAUČNOM VEĆU
UNIVERZITETA U BEOGRADU- FARMACEUTSKOG FAKULTETA
TO THE ACADEMIC COUNCIL
UNIVERSITY OF BELGRADE- FACULTY OF PHARMACY

KOMISIJI ZA POSLEDIPLOMSKE STUDIJE
TO THE COMMITTEE FOR POSTGRADUATE STUDIES

Nastavno-naučno Veće Farmaceutskog fakulteta, Univerziteta u Beogradu, na sednici održanoj 17.6.2021. godine imenovalo je Komisiju za ocenu i odbranu završene doktorske disertacije kandidata magistra farmacije Jelene Petrović, pod naslovom:

Uticaj magnezijuma na ponašanje, neuroendokrine i promene na miokardu uzrokovane hiperaktivnošću osovine hipotalamus-hipofiza-nadbubreg kod pacova

The Academic Council of the Faculty of Pharmacy, University of Belgrade has nominated the Committee for evaluation and defense of doctoral dissertation of Jelena Petrović, master of pharmacy, at the meeting held on June 17th, 2021, entitled:

Effects of magnesium on behavior, neuroendocrine and changes in myocardium induced by hyperactivity of the hypothalamic-pituitary-adrenal axis in rats

Komisija u sastavu/ *Following members of the Committee:*

1. Dr Vesna Pešić, mentor rada, redovni profesor, Univerzitet u Beogradu-Farmaceutski fakultet*/ supervisor, full time professor, University of Belgrade-Faculty of Pharmacy*

2. Dr Magnus Ingelman-Sandberg, gostujući profesor, Univerzitet u Beogradu-Farmaceutski fakultet i profesor, Karolinska Institut, Stokholm*/ visiting professor, University of Belgrade-Faculty of Pharmacy and full time professor, Karolinska Institutet, Stockholm*

3. Dr Milica Labudović-Borović, vanredni profesor, Univerzitet u Beogradu-Medicinski fakultet*/ associate professor, University of Belgrade-Faculty of Medicine*

4. Dr Bojana Pejušković, docent, Univerzitet u Beogradu – Medicinski fakultet/ *assistant professor, University of Belgrade-Faculty of Medicine*

5. Dr Marin Jukić, docent, Univerzitet u Beogradu – Farmaceutski fakultet/ *assistant professor University of Belgrade-Faculty of Pharmacy*

pregledala je priloženu disertaciju i podnosi Nastavno-naučnom veću Farmaceutskog fakulteta, univerziteta u Beogradu sledeći/*Based on detailed review of the submitted dissertation, present to the Academic Council of the Faculty of Pharmacy-University of Belgrade the following*

IZVEŠTAJ/*REPORT*

A. PRIKAZ SADRŽAJA DOKTORSKE DISERTACIJE/ *THE CONTENT OF THE DOCTORAL DISSERTATION*

Doktorska disertacija kandidata Jelene Petrović pod naslovom **" Uticaj magnezijuma na ponašanje, neuroendokrine i promene na miokardu uzrokovane hiperaktivnošću osovine hipotalamus-hipofiza-nadbubreg kod pacova"** napisana je jasnim i preglednim stilom na 142 strane teksta, formata A4, fontom *Times New Roman*, veličine 12 i jednostrukim proredom. U sklopu disertacije prikazano je ukupno 10 tabela, 35 slika i 424 literaturnih navoda. Disertacija sadrži sledeća poglavlja: Uvod (21 strana), Ciljeve rada (2 strane), Materijal i metode (17 strana), Rezultate (29 strana), Diskusiju (40 strana), Zaključak (2 strane) i Literaturu (31 strana). Na početku disertacije predstavljen je Sažetak rada na srpskom i engleskom jeziku, dok se na kraju nalazi prilog (Lista skraćenica i oznaka), kratka biografija kandidata, i potpisane izjave kandidata o autorstvu, istovetnosti štampane i elektronske verzije i korišćenju doktorske disertacije.

*Doctoral dissertation of the candidate Jelena Petrović entitled " **Effects of magnesium on behavior, neuroendocrine and changes in myocardium induced by hyperactivity of the hypothalamic-pituitary-adrenal axis in rats"** is written in a clear and concise style on 142 pages, by using A4 text format, Times New Roman font, size 12 and single spacing. The dissertation includes 10 tables, 35 figures and 424 references and comprises the following chapters Introduction (21 pages), Research aims (2 pages), Materials and methods (17 pages), Results (29 pages), Discussion (40 pages), Conclusion (2 pages) and References (31 pages). At the beginning of the dissertation, an abstract is presented in Serbian and English,*

while at the end of the dissertation the following material is included: attachment (List of abbreviations and designations), the author's biography and signed declarations on authorship, the complete similarity between electronic and printed version of the dissertation and copyright transfer.

Uvodno poglavlje se sastoji iz tri dela: (1) Magnezijum, (2) Depresivni poremećaj i hipotalamo-hipofizno-adrenalna osovina (*hypothalamic-pituitary-adrenal axis, HPA*) i (3) Depresivni poremećaj i kardiovaskularna oboljenja, u sklopu kojih su prikazana dosadašnja naučna saznanja vezana za temu same disertacije. U prvom delu uvoda, objašnjena je homeostaza i fiziološki značaj magnezijuma (Mg), u humanom organizmu. Opisani su transportni molekuli uključeni u intestinalnu apsorpciju Mg, bubrežnu ekskreciju, kao i proces mobilizacije iz kostiju. Potom su objašnjene fiziološke uloge Mg i njegov značaj u očuvanju zdravlja nervnog i kardiovaskularnog sistema. Nakon toga, dat je osvrt na referentne vrednosti za preporučen dnevni unos Mg, izazove koji se javljaju prilikom određivanja koncentracije Mg u serumu/plazmi, i predstavljeni su uzroci, kao i simptomi hipomagnezemije i hipermagnezemije. Na kraju ovog dela, prikazani su literaturni podaci koji ukazuju na modulatorni efekat magnezijuma na aktivnost osovine hipotalamus-hipofiza-nadbubrežna žlezda i istaknuta je potencijalna uloga ovog bioelementa u etiologiji i neurobiologiji depresivnog poremećaja. U drugom delu, u okviru razmatranja depresivnog poremećaja i HPA osovine, najpre je objašnjen odgovor HPA sistema na stres, emotivne ili fizičke prirode, kao i mehanizam negativne povratne sprege, preko kog glukokortikoidi inhibiraju oslobađanje kortikotropin-oslobađajućeg faktora/hormona (eng. *corticotropin releasing factor, CRF*) i adrenokortikotropnog hormona (eng. *adrenocorticotropic hormone, ACTH*) i na taj način, smanjuju sopstvenu sintezu i sekreciju. Potom je istaknuta uloga HPA ose u regulaciji afektivnog ponašanja i činjenica da se u velikom broju studija hiperaktivnost HPA osovine navodi kao neurobiološki supstrat u osnovi depresivnog poremećaja. Usledilo je razmatranje epidemioloških podataka o depresivnom poremećaju, detaljan opis kliničke slike i farmakoterapije depresije. Nakon toga, objašnjena je veza koja postoji između HPA osovine, imunskog sistema i depresivnog poremećaja. Takođe, pojašnjeno je da se kod osoba koje pate od depresije, pored hiperaktivnosti HPA osovine, javlja i supresija procesa adultne neurogeneze u hipokampusu. Na kraju ovog dela Uvoda, dat je osvrt na animalne modele depresivnog ponašanja i predstavljeni su literaturni podaci vezani za model depresije koji je implementiran u istraživanje sprovedeno u okviru doktorske disertacije kandidata. Naime, u okviru istraživanja, primenjen je prethodno validiran model koji se zasniva na dugotrajnoj

primeni ACTH kod glodara i koji se danas smatra modelom depresije rezistentne na primenu tricikličnih antidepresiva. U trećem delu Uvoda, objašnjeni su neurobiološki mehanizmi koji povezuju depresivni poremećaj i kardiovaskularna oboljenja, kao i uloga hroničnog stresa, praćenog povišenim nivoom cirkulišućeg kortizola, kao nezavisnog faktora rizika, u razvoju srčanih oboljenja. Na kraju Uvodnog dela, predstavljeni su *in vitro* modeli ćelijskih kultura kardiomiocita, koji imaju veliki značaj u izučavanju patoloških mehanizama u osnovi srčanih oboljenja, ispitivanju kardiotoksičnog potencijala i razvoju novih terapijskih pristupa. Takođe, istaknut je značaj i prednost primene trodimenzionalnih modela, uključujući i model sferoida, u odnosu na tradicionalne dvodimenzionalne ćelijske kulture. Naime, studije su pokazale da 3D modeli sferoida humanih kardiomiocita omogućavaju produženu vijabilnost ćelija u kulturi, u odnosu na 2D modele, i da verno reflektuju morfološke i funkcionalne fenotipske osobine humanih srčanih mišićnih ćelija.

*The **Introduction** comprises three sections: 1) Magnesium (Mg), 2) Major depressive disorder and hypothalamic-pituitary-adrenal (HPA) axis and 3) Depression and Cardiovascular disease, encompassing literature data relevant to the research topic. In the first section, regulation of magnesium balance and its physiological roles in the human body are presented. Transport pathways and proteins involved in intestinal absorption and renal excretion, and bones, the main Mg storage system of the body, are described in detail. Next, important physiological roles of Mg in maintaining proper functions of the nervous and cardiovascular systems are considered. Subsequently, the recommended daily allowance (RDA) for magnesium and an average daily intake are compared and challenges in determining Mg status in the human body are introduced. Moreover, causes, signs and symptoms of hypo- and hypermagnesemia, are listed and explained, in detail. Importantly, modulatory effects of Mg on HPA axis function and the role of Mg in neurobiology of depression are described. In the second section, physiological response of HPA axis to both physical and emotional stress is described, including the negative feedback mechanism that controls glucocorticoid secretion. Glucocorticoids inhibit the release of corticotropin releasing factor (CRF) and adrenocorticotropic hormone (ACTH), and thus reduce their own synthesis and secretion. Afterwards, prominent role of HPA axis in the regulation of mood and emotional behavior is depicted. A vast number of studies have shown that hyperactivity of the HPA axis is one of the major findings in neurobiology of depressive disorder. Next, epidemiological data, clinical signs and symptoms, as well as pharmacotherapy for depression, are presented. Subsequently, the relationship between HPA stress axis, immune system and depressive disorder is described in detail. Importantly, in patients suffering from*

depression major neurobiological alterations involve hyperactivity of HPA axis and suppression of adult hippocampal neurogenesis. These findings are considered in great detail in the Introduction section. In addition, animal models of depression are listed and findings related to ACTH-induced model of depression resistant to tricyclic antidepressants are presented. Namely, studies have shown that protracted ACTH treatment in rodents evokes neuroendocrine and behavioral changes that reflect depressive phenotype that does not respond to administration of tricyclic antidepressants. ACTH-induced model of depression was employed in the dissertation research. In the third section, neurobiological mechanisms underlying depression and cardiovascular disease are overviewed. Furthermore, major role of chronic stress accompanied by elevated glucocorticoid levels, an independent risk factor for development and progression of heart disease, is considered. Lastly, in the Introduction section, cell cultures of adult cardiomyocytes as models, for studying cardiopathological mechanisms and signaling pathways, are presented. These model systems are routinely employed in drug development for cardiotoxic liability testing of novel drug candidates. Conventional 2D monolayer cultures have numerous limitations, including lack of sensitivity and accuracy in predicting toxicity, and in the past decade focus has been shifted to new emerging 3D in vitro models, which more faithfully resemble human cardiac phenotypes and functions.

Ciljevi istraživanja su jasno definisani – ispitivanje efekata peroralne primene Mg (300 mg/L vode za piće, 28 dana, u obliku magnezijum sulfata) na bihejvioralne, parametre aktivnosti HPA osovine i adultne neurogeneze u hipokampusu, u modelu depresivnog ponašanja, rezistentnog na triciklične antidepresive i indukovanog ponavljanom primenom ACTH (10 μg/dan, 21 dan, *s.c.*) kod mužjaka Wistar pacova. Pored toga, cilj je bio ispitivanje kardioprotektivnih efekata magnezijuma, kao i uticaja na proliferaciju kardiomiocita, u datom modelu. Finalno, cilj je bio razviti i implementirati trodimenzionalni *in vitro* model sferoida kardiomiocita i odrediti potencijalne direktne efekte ACTH na humane kardiomiocite u kulturi. Ciljevi istraživanja u okviru ove doktorske disertacije su realizovani kroz tri eksperimentalne faze.

*The scientific research of this dissertation has three **aims** which are clearly defined. The first aim was to investigate the effects of protracted Mg treatment (300 mg/L, via drinking water, 28 days) on behavior, neuroendocrine changes and parameters of hippocampal neurogenesis in a model of depressive-like behavior induced by chronic administration of*

adrenocorticotropic hormone (10 μg/day, 21 days) and resistant to tricyclic antidepressants. The second aim was to examine the effects of protracted administration of ACTH in rats on parameters of proliferation and fibrosis in myocardium, as well as potential cardioprotective effects of Mg. The third objective was to develop and optimize culture conditions in a 3D in vitro model of human cardiomyocytes in order to assess potential direct effects of ACTH on cardiomyocytes. To achieve this set of objectives, the research comprised of three phases.

U poglavlju **Materijal i metode,** opisan je dizajn istraživanja, navedeni su svi korišćeni reagensi i aparatura i detaljno je prikazana metodologija primenjena u istraživanju.

U prvoj fazi istraživanja, mužjaci pacova Wistar soja su podeljeni u četiri grupe koje su bile izložene sledećim tretmanima: Mg rastvoren u vodi za piće (300 mg/L, 28 dana, *per os*), supkutane injekcije ACTH (10 μg/dan, 21 dan, *s.c.*), ACTH/Mg kombinovani tretman (primena ACTH tokom poslednje tri nedelje Mg tretmana) i kontrolna grupa - fiziološki rastvor (400 μL/dan, 21 dan, *s.c.*). U prvoj fazi istraživanja, u modelu depresivnog ponašanja indukovanog ACTH tretmanom, ispitivani su efekti primene magnezijuma na ponašanje eksperimentalnih životinja, koncentraciju kateholamina i serotonina, hormona neuroendokrinog HPA sistema – ACTH i kortikosterona, kao i nivo IL-6, u plazmi. Takođe, prilikom izlaganja životinja akutnom stresu, u vidu testa forsiranog plivanja (FST), praćen je odgovor HPA ose i oslobađanje kateholamina i serotonina. Pored toga, izvršena je analiza parametara adultne neurogeneze u hipokampusu.

*In the **Materials and methods** section, all materials and reagents that were used are listed and all the protocols are described in detail.*

In the first phase, animals were randomly assigned into one of four groups. Mg group received Mg dissolved in drinking water (300 mg/L, per os) for 28 days. ACTH group received subcutaneous ACTH injections (10 μg/400 μL/day) for 21 days and drank tap water for 28 days. ACTH/Mg group was treated with ACTH for 21 days and Mg dissolved in drinking water for 28 days. Animals from the Control group received subcutaneous saline injections (400 μL/ day) for 21 days and drank tap water for 28 days.

In the first phase, in a model of HPA axis hyperactivity induced by repeated application of ACTH in rats and previously validated as a model of depression-like behavior, the effects of Mg on behavior, parameters of HPA axis activity and hippocampal neurogenesis were investigated. In the blood, following parameters that reflect the activity of HPA axis were determined: ACTH, corticosterone levels, as well as the concentration of IL-6, a

proinflammatory cytokine closely interacting with HPA axis. Furthermore, plasma levels of adrenaline, noradrenaline and serotonin were measured. In addition, the effects of acute stress evoked by FST exposure, as well as the influence of ACTH and Mg treatment were monitored. Moreover, the expression of BDNF and Ki-67 was assessed in rats' hippocampal neurons, as important molecular determinants of neurogenesis.

U drugoj fazi istraživanja, životinje su takođe podeljene u četiri eksperimentalne grupe i primenjeni su prethodno navedeni tretmani, ali s ciljem određivanja markera proliferacije i fibroze miokarda. U ovoj fazi, tokom Mg suplementacije, praćena je proliferacija kardiomiocita, fibroblasta i vaskularnih endotelnih ćelija u srcu, ekspresija anti-apoptotskog markera u endotelnim ćelijama, promena dijametra kardiomiocita i deponovanje endomizijalnog kolagena, u modelu depresivnog ponašanja indukovanog ACTH tretmanom. Takođe, u ovoj fazi istraživanja, izvršena je izolacija prefrontalnog korteksa u cilju analize sadržaja bioelemenata – Fe, Zn, Mg i Cu. U istraživanja obuhvaćena ovom doktorskom disertacijom uključeno je ukupno 100 mužjaka pacova *Wistar* soja, starih osam nedelja na početku eksperimenta.

In the second phase, animals were randomly divided into four treatment groups. Mg group received Mg dissolved in drinking water (300 mg/L, 28 days). ACTH group received subcutaneous ACTH injections (10 μg/400 μL/day, 21 days). Animals from the ACTH/Mg group were treated with ACTH for 21 days and Mg dissolved in drinking water for 28 days and Control group received subcutaneous saline injections (400 μL/ day, 21 days). In the second phase, the focus was on the effects of protracted administration of ACTH on parameters of cardiomyocyte, fibroblast and vascular endothelial cell proliferation and potential cardiprotective effects of Mg. Additionally the expression of Bcl-2, an anti-apoptotic protein in vascular endothelial cells was assessed. Furthermore, cardiomyocyte diameter was analyzed and endomysial collagen deposition was assessed. Lastly, prefrontal cortex of male Wistar rats was isolated and levels of bioelements were determined.

One hundred animals – male Wistar rats, eight weeks old at the onset, were involved in this research.

U trećoj fazi istraživanja, nakon izbora odgovarajućeg medijuma i uslova kultivacije, implementiran je 3D model sferoida kardiomiocita formiranih indukcijom humanih pluripotentnih stem ćelija. Izbor se zasnivao na praćenju vijabilnosti kardiomiocita,

identifikaciji proteina miofibrila imunofluorescentnom metodom i analizi ekspresije gena: *MYL2*, *MYL7*, *PLN* i *NKX2.5*. Potom su 3D sferoidi kardiomiocita izloženi fiziološkim (22 ng/L; 1x ACTH) i suprafiziološkim (660 ng/L; 30x ACTH) koncentracijama ACTH, i nakon 7, 14 i 19 dana tretmana, analizirani su sledeći parametri: 1) vijabilnost ćelija, 2) nivo aktivirane kaspaze 3 i 3) genska ekspresija.

In the third phase, a 3D in vitro model of human cardiomyocyte spheroids was established, including culture conditions that provide cell viability and preserved phenotype for several weeks in the culture. For this purpose, cell viability and specific gene expression (MYL2, MYL7, PLN i *NKX2.5) were evaluated and immunohistochemical detection of F-actin and sarcomeric α-actinin were performed. This model was employed in examining in vitro potential direct effects of physiological (22 ng/L; 1x ACTH) and supraphysiological concentrations (660 ng/L; 30x ACTH) of ACTH on human cardiomyocyte viability, cleaved caspase 3 and relevant gene expression.*

Prilikom ispitivanja promena u ponašanju eksperimentalnih životinja korišćeni su test otvorenog polja (eng. Open Field Test, OFT) i test forsiranog plivanja (eng. Forced Swim Test, FST). Postupak izvođenja navedenih bihejvioralnih testova, kao i parametri koji su određivani, detaljno su objašnjeni u ovom poglavlju doktorske disertacije. Koncentracija kateholamina i serotonina u plazmi eksperimentalnih životinja analizirana je primenom metodom tečne hromatografije pod visokim pritiskom sa elektrohemijskim detektorom (eng. High Performance Liquid Chromatography-Electrochemical Detector, HPLC-ECD), dok je nivo kortikosterona praćen korišćenjem metode tečna hromatografija- elektrosprej jonizacija-tandem masena spektrometrija (eng. Liquid Chromatography-Electrospray Ionisation–Tandem mass spectrometry, LC-ESI-MS/MS). Koncentracija ACTH, kao i proinflamatornog citokina IL-6, u plazmi, određivana je primenom ELISA (enzyme-linked immunosorbent assay) metode. U okviru ovog dela poglavlja, detaljno su opisani princip i karakteristike navedenih metoda. Parametri adultne neurogeneze su ispitivani primenom imunohistohemijske metode detekcije i rezultati su izraženi kao broj 1) Ki-67 i 2) BDNF imunopozitivnih ćelija, po površini vidnog polja, u dentatnom girusu hipokampusa. Izolacija moždanog tkiva, fiksacija, dehidratacija i kalupljenje u parafinu, a kasnije i rehidratacija i sam postupak bojenja, uključujući i primenjena razblaženja antitela, detaljno su prikazani. Prilikom određivanja koncentracije bioelemenata – Fe, Cu, Zn i Mg, u prefrontalnom korteksu (PFC) životinja, primenjena je metoda plamene atomske apsorpcione spektrofotometrije. U

ovom delu je predstavljen detaljan opis pripreme uzorka i same procedure. Praćenje promena u dijametru kardiomiocita i analiza količine deponovanog endomizijalnog kolagena, izvršeni su primenom metode koju su uveli Gordon i Sweets, bojenjem retikulinskih vlakana u srcu. Analiza parametara proliferacije – Ki-67 i nuklearnog antigena proliferišućih ćelija (eng. proliferating cell nuclear antigen, PCNA), kao i anti-apoptotskog proteina Bcl-2 izvršena je primenom imunohistohemijske metode. U ovom delu je dat detaljan opis izolacije miokarda eksperimentalnih životinja, fiksacije, dehidratacije, kalupljenja u parafinu, a kasnije i rehidratacije, postupka bojenja i kvantifikacije. U *in vitro* modelu sferoida humanih kardiomiocita, ispitivanje vijabilnosti ćelija, praćenjem nivoa ATP-a, kao i merenje vrednosti aktivirane kaspaze-3, markera apoptoze u kulturi, sprovedeni su primenom metode luminiscencije. Karakteristike ove metode i sam postupak su detaljno prikazani. Takođe, u 3D modelu sferoida kardiomiocita, prilikom ispitivanja fenotipskih osobina, određivana je ekspresija sledećih gena: *MYL2*, *MYL7*, *PLN*, *NKX2.5* i *Ki-67*, metodom lančane reakcije polimerizacije u realnom vremenu (eng. Real Time–Polymerase Chain Reaction, RT–PCR). Proces izolacije iRNK, sinteza komplementarne DNK i postupak RT-PCR analize jasno su prikazani. Dodatno, za detekciju i analizu proteina F-aktina i α-aktinina u sferoidima humanih kardiomiocita korišćena je imunofluorescentna tehnika i konfokalni mikroskop i jasno je opisan postupak ekstrakcije sferoida, fiksacije i bojenja.

Behavioral changes in rats were evaluated in standard behavioral tests: open field test (OFT) and FST. Test procedures and all parameters that were analyzed, are described in detail in this section of dissertation. Plasma levels of catecholamines and serotonin were determined using Reversed Phase High Pressure Liquid Chromatography with an Electrochemical Detector (RP-HPLC-ECD), whereas corticosterone levels were measured with Liquid Chromatography-Electrospray Ionization-Tandem mass spectrometry (LC-ESI-MS/MS) system. Concentrations of ACTH and IL-6 in plasma were determined using an enzyme-linked immunosorbent assay (ELISA). All relevant characteristics of the applied methods, preparation and storage of plasma samples are described in detail. Immunohistochemical staining of brain tissue sections was used to investigate changes in adult hippocampal neurogenesis and number of BDNF and Ki-67 immunopositive cells in this brain region was determined. All details related to dehydration, paraffin embedding, staining procedure, including antibody dilutions, and quantification are presented in this section of the dissertation.

Levels of Zn, Cu, Fe and Mg in the PFC were assessed by flame atomic absorption spectrophotometry. In this section, sample preparation and analytical method are provided, in detail. Assessment of cardiomyocyte width and interstitial reticulin fibrosis was performed by using reticulin staining, according to Gordon and Sweets' method. Immunohistochemistry was applied to investigate changes in markers of proliferation Ki-67 and proliferating cell nuclear antigen (PCNA), and anti-apoptotic protein Bcl-2 in the heart. All relevant details related to cardiac tissue preparation, dehydration, paraffin embedding, staining procedure, including antibody dilutions, and quantification are included in this section of the dissertation.

Cell viability in the 3D in vitro model system was determined by measuring ATP levels of single human induced pluripotent stem cell (iPSC)-derived cardiomyocyte spheroid. Cell viability and caspase-3 activity were examined by measuring luminescence. In both assays, the protocol complied with manufacturer's instructions and the description is included in this section. Analysis of gene expression - MYL2, MYL7, PLN, NKX2.5 i Ki-67 in 3D cardiomyocyte spheroids was performed by using Quantitative Real Time–Polymerase Chain Reaction (RT-qPCR). The protocols for mRNA isolation and cDNA synthesis, as well as primers' characteristics, are described in detail. Immunohistochemical analysis of iPSC-derived cardiomyocyte spheroids and detection of F-Actin and α-Actinin are presented in this section of dissertation.

U delu Statistička obrada rezultata, navedene su sve statističke metode koje su bile korišćene: jednofaktorska, dvofaktorska i trofaktorska ANOVA, praćene odgovarajućom post-hoc analizom, Spirmanova korelacija i Studentov t test. Statistička analiza rezultata prve i druge faze istraživanja izvršena je primenom softvera PASW Statistics, verzija 18 (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.), dok je za obradu podataka iz treće faze korišćen Microsoft Excel 2010.

One-way, Two-way and Three-way ANOVA, followed by the corresponding post hoc test, were used for data analysis. In addition Spearman's correlation and heteroscedastic 2-tailed Student's t tests were performed. SPSS package PASW Statistics 18 (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago: SPSS Inc.) and Microsoft Excel 2010 were used for statistical analysis.

U poglavlju **Rezultati** predstavljeni su originalni rezultati dobijeni u sklopu istraživanja ove doktorske disertacije. Dat je detaljan opis i rezultati su prikazani grafički i tabelarno, kroz 20 slika i 6 tabela.

*In the **Results** section, all the results obtained in the experimental work of the dissertation are included and described in detail. Results are shown as figures and tables, encompassing 20 figures and 6 tables.*

U poglavlju **Diskusija** prikazana je detaljna analiza rezultata dobijenih u okviru ove disertacije, u kontekstu dostupnih literaturnih podataka.

*In the **Discussion** section, a comprehensive analysis of the results obtained in the experimental work of the dissertation and overview of the available literature data, related to the research topic, are provided.*

U sklopu poglavlja **Zaključak** predstavljeni su svi relevantni zaključci koji proističu iz rezultata sprovedenog istraživanja i njihove analize.

*In the **Conclusion** section, all relevant conclusions are presented and analysed.*

Na kraju doktorske disertacije nalazi se poglavlje **Literatura,** u kom su navedene sve reference (ukupno 424) koje su korišćene tokom izrade ove doktorske disertacije.

***References** section is included at the end of the dissertation and contains all the references (424 in total) that were cited in the text.*

B. OPIS POSTIGNUTIH REZULTATA/ *DESCRIPTION OF THE OBTAINED RESULTS*

Rezultati istraživanja su prikazani u okviru 3 veće i 11 manjih celina. U prvoj većoj celini, predstavljeni su rezultati prve faze istraživanja. Najpre je objašnjen uticaj ACTH i Mg tretmana na telesnu i masu nadbubrežne žlezde eksperimentalnih životinja. Dobijeni rezultati ukazuju da se u primenjenom modelu depresivnog ponašanja rezistentnog na triciklične antidepresive, usled dugotrajne izloženosti ACTH, kod životinja javlja smanjenje telesne mase i hipertrofija adrenalnih žlezda. Hronična Mg suplementacija putem vode za piće je inhibirala ovaj efekat ACTH na telesnu masu mužjaka Wistar pacova, međutim, Mg nije

sprečio razvoj hipertrofije nadbubrežne žlezde, koja se ogleda u primetnom porastu apsolutne i relativne mase žlezde. U ovoj fazi je praćeno ponašanje eksperimentalnih životinja primenom odgovarajućih testova - OFT i FST. Pokazano je da životinje izložene hroničnom ACTH tretmanu odlikuje ponašanje koje se može uporediti sa simptomima anksioznosti kod ljudi, koje se kod mužjaka pacova manifestovalo skraćenim vremenom provedenim u centralnoj zoni polja za izvođenje OFT, kao i primetnim smanjenjem pređenog puta u ovoj zoni. Pored toga, ACTH tretman je doveo do razvoja depresivnog fenotipa, koji se ogleda u izraženom povećanju vremena imobilnosti i skraćenju vremena provedenog u plivanju, tokom izvođenja FST. Primena Mg je ostvarila anksiolitički i antidepresivni efekat kod mužjaka Wistar pacova, u modelu depresije rezistentne na triciklične antidepresive. Naime, Mg je doveo do primetnog porasta vremena provedenog u centralnoj zoni tokom OFT, dok je u testu forsiranog plivanja primećeno značajno skraćenje perioda imobilnosti, praćeno porastom vremena provedenog u plivanju u ACTH/Mg grupi u poređenju sa ACTH životinjama. U okviru ove faze istraživanja, ispitivan je efekat hronične primene ACTH i Mg, kao i akutnog stresa indukovan izlaganjem glodara FST, na nivo kateholamina i serotonina, kao i na parametre aktivnosti HPA ose - ACTH, kortikosteron i IL-6, u plazmi. Dobijeni rezultati ukazuju da Mg tretman dovodi do izraženog porasta nivoa adrenalina, noradrenalina i serotonina prilikom izlaganja životinja akutnom stresu. Takođe, akutni stres je uzrokovao značajan porast koncentracije adrenalina, u svim grupama, izuzev kod ACTH životinja, i noradrenalina u Mg i ACTH/Mg grupi. Primena ACTH kod glodara je indukovala upadljiv porast nivoa kortikosterona u plazmi, što oslikava hiperaktivnost HPA osovine. U ACTH/Mg grupi, hronična primena Mg je indukovala pad koncentracije ACTH i cirkulišućeg proinflamatornog citokina IL-6 nakon izlaganja akutnom stresu, kao i smanjenje nivoa kortikosterona kod nestresiranih životinja. Rezultati imunohistohemijske analize markera proliferacije Ki-67 u hipokampusu upućuju da i ACTH i Mg tretman deluju stimulatorno na proliferaciju neurona, dok njihova kombinovana primena ima sinergistički efekat. Sa druge strane, samo Mg je indukovao povećanje ekspresije neurotrofina BDNF - drugog markera neurogeneze koji je ispitivan, i zabeležen je značajan porast broja BDNF imunopozitivnih neurona kod ACTH/Mg životinja u odnosu na ACTH grupu.

The results are arranged in three major sections and eleven sub-sections. In the first section, results of the first experimental phase of dissertation are displayed. At the beginning of this chapter, the effects of ACTH and Mg treatment on body and adrenal glands' weight are presented. The results show that the body weight was significantly lower in ACTH-treated animals relative to controls, while Mg treatment prevented such loss of the body weight in

ACTH/Mg group. ACTH evoked adrenal hypertrophy, manifesting in a significant increase in the absolute and relative weight of adrenal gland. Chronic Mg supplementation could not reverse this effect on adrenal glands. Next, behavioral changes were assessed in this phase, and for this purpose OFT and FST were performed. Animals that were exposed to chronic ACTH treatment showed reduced time spent in the central zone and decreased distance traveled in this zone, thus displaying anxiety-like behavior. Mg increased the time spent in the central zone in ACTH/Mg relative to ACTH group. In the FST, ACTH animals spent more time being immobile compared to controls, indicating despair-like behavior, while Mg reversed this effect and reduced the immobility in ACTH/Mg group relative to ACTH group. Moreover, ACTH animals spent less time swimming in comparison to controls and ACTH/Mg animals. In this phase, the effects of ACTH and Mg treatment, as well as of the FST-evoked stress on plasma levels of ACTH, corticosterone and IL-6, parameters indicative of HPA axis activity were analyzed. Results revealed that Mg treatment induces a prominent increase in adrenaline, noradrenaline and serotonin levels in plasma, during acute stress exposure. Additionaly, FST-induced stress led to significant increase in plasma adrenaline in all groups, except for ACTH animals. Moreover, following acute stress an increase in plasma noradrenaline was observed only in Mg and ACTH/Mg group. Results showed chronic ACTH treatment in rats evokes HPA axis hyperactivity, manifesting in elevated plasma corticosterone levels. Importantly, Mg treatment significantly decreased plasma ACTH and IL-6 levels in rats, following acute stress exposure. In non-stressed animals Mg significantly reduced plasma corticosterone levels in ACTH/Mg relative to ACTH group. Immunohistochemical analysis revealed that both ACTH and Mg treatments induced an increase in the number of Ki-67 positive cells in the hippocampus. Combined administration showed synergistic effect and in ACTH/Mg animals there were significantly more Ki-67 immunopositive cells than in both Mg and ACTH group. Importantly, Mg treatment evoked an increase in the number of BDNF immunopositive cells. Increase in both Ki-67 and BDNF expression strongly suggests enhanced hippocampal neurogenesis following Mg exposure in male rats.

U okviru poglavlja Rezultati, u drugoj većoj celini, prikazani su rezultati druge faze istraživanja. Naime, u drugoj eksperimentalnoj fazi, tokom ispitivanja promena u homeostazi biometala u PFC, zabeležen je pad koncentracije Mg nakon ACTH tretmana za 23%, dok je primena magnezijuma indukovala porast nivoa Mg kod ACTH/Mg životinja za 22,3% u

odnosu na ACTH grupu. Date vrednosti vezane za promene nivoa Mg u PFC nisu dostigle statističku značajnost. Međutim, hronična Mg suplementacija je indukovala značajan porast koncentracije Cu, dok je ACTH izazvao pad nivoa Cu u PFC. Nisu uočene promene u sadržaju Zn i Fe u PFC kod mužjaka Wistar pacova, nakon ACTH i/ili Mg tretmana. Prilikom ispitivanja uticaja ACTH na srce u modelu depresivnog ponašanja rezistentnog na triciklične antidepresive, primećen je izraženi porast proliferacije fibroblasta i vaskularnih endotelnih ćelija, koji se ogleda u povećanju broja Ki-67 imunopozitivnih ćelija u miokardu. Hronična primena Mg je ostvarila kardioprotektivno dejstvo i suprimirala navedeni efekat ACTH. Pored toga, pokazano je da ACTH indukuje porast ekspresije anti-apoptotskog markera Bcl-2 u vaskularnim endotelnim ćelijama mikrocirkulacije srca, dok Mg inhibira ovaj efekat. U ovoj fazi je takođe otkriveno da ponavljana aplikacija ACTH kod eksperimentalnih životinja izaziva povećano deponovanje endomizijalnog kolagena, što zajedno sa porastom proliferacije fibroblasta i vaskularnih endotelnih ćelija, snažno upućuje na razvoj fibroze miokarda u ACTH grupi. Dugotrajna Mg suplementacija je inhibirala pomenuti efekat ACTH i razvoj fibroze u ACTH/Mg grupi. Rezultati dobijeni analizom nuklearnog proteina Ki-67 u srcu, u sklopu ove faze, ukazuju na stimulatorni efekat i ACTH i Mg tretmana na proliferaciju kardiomiocita kod mužjaka Wistar pacova. Pored opisanih promena u srcu eksperimentalnih životinja, primećeno je da i ACTH i Mg tretman dovode do značajnog smanjenja transnuklearnog dijametra kardiomiocita, dok je neočekivano, kombinovana ACTH/Mg primena za posledicu imala normalizaciju ovog parametra.

In the second section of this chapter, results of the second experimental phase are presented. Namely, in this research phase, effects of ACTH and Mg treatments on the levels of bioelements – Fe, Zn, Cu and Mg were assessed in rat prefrontal cortex. Results showed that ACTH and Mg treatment, as well as their interaction, had no significant effects on Fe, Zn and Mg concentrations in the PFC in rats. However, data showed that Mg concentration in the PFC was reduced by 23% following ACTH exposure in comparison to Controls. On the other hand, Mg co-administration to ACTH evoked also non-significant but apparent increase by 22.3% of PFC Mg level in ACTH/Mg rats compared to ACTH group. In addition, ACTH treatment evoked a significant decrease in Cu levels in the PFC, whereas Mg supplementation led to an increase in Cu concentration.

Importantly, in this phase, the impact of stress on myocardial health was evaluated. Results showed that in a model of depressive-like behavior, resistant to tricyclic antidepressants, ACTH evokes a prominent increase in fibroblast and vascular endothelial cell proliferation in

the heart, manifesting in increased expression of Ki-67 in these cells. Chronic Mg supplementation exerted cardioprotective effects, by suppressing proliferative response in cardiac fibroblasts and vascular endothelial cells. Next, increase in levels of the anti-apoptotic marker Bcl-2 in endothelial cells in the hearts of ACTH-treated rats was observed. On the other hand, magnesium inhibited this effect. Significant increase in cardiac fibroblast and endothelial cell proliferation, accompanied by increase in type III collagen deposition in ACTH-treated animals compared to the control group, indicates that long-term exposure to ACTH resulted in the development of cardiac fibrosis. Importantly, Mg treatment ameliorated ACTH-induced cardiac fibrosis and promoted cardiomyocyte proliferation in male rats. Furthermore, both ACTH and Mg treatments enhanced cardiomyocyte proliferation in male rats. Additionlly, both ACTH and Mg treatments led to a decrease in cardiomyocyte diameter in rats. However, in rats chronically exposed to combined ACTH and Mg treatment, cardiomyocyte width was normalized and showed no differences compared to the control group.

U trećem delu ovog poglavlja disertacije, prikazani su rezultati treće eksperimentalne faze. Nakon razvoja i implementacije 3D modela sferoida humanih kardiomiocita, uporednom analizom vijabilnosti ćelija i ekspresije relevantnih gena u različitim medijumima za ćelijske kulture, ispitivan je *in vitro* uticaj fiziološke (22 ng/L; 1x ACTH) i suprafiziološke koncentracije (660 ng/L; 30x ACTH) ACTH na srčane mišićne ćelije. Dobijeni rezultati su pokazali značajan porast nivoa ATP u sferoidima kardiomiocita, u odsustvu proliferacije, nakon izlaganja visokim koncentracijama ACTH. Nivo aktivirane kaspaze - 3, markera apoptoze, je ostao nizak u kulturi, nakon izlaganja 1x ACTH i 30x ACTH. Takođe, nije bilo značajne razlike u ekspresiji *MYL2*, *PLN*, kao ni *NKX2.5* u kardiomiocitima sferoida izloženim dugotrajnom tretmanu sa 1x ACTH, kao i 30x ACTH, u poređenju sa netretiranim ćelijama.

In the third section of this chapter, results of the third experimental phase are depicted. A 3D spheroid model of human cardomyocytes was developed and potential in vitro effects of physiological (22 ng/L; 1x ACTH) and supraphysiological ACTH levels (660 ng/L; 30x ACTH) were investigated. In vitro study revealed that a high dose of ACTH provoked increases in cellular ATP levels in cardiomyocyte spheroids, which in the absence of proliferation could be due to increases in cell size. Next, cleaved caspase 3 determination, indicative of programmed cell death, revelead very low levels of apoptosis throughout the

culture period, following exposure to both 1x ACTH and 30x ACTH. Lastly, protracted ACTH treatment did not provoke drastic changes in the expression of MYL2, PLN, NKX2.5 and Ki-67 over time in iPSC-derived cardiomyocyte spheroids.

C. UPOREDNA ANALIZA REZULTATA DOKTORSKE DISERTACIJE SA PODACIMA IZ LITERATURE/ COMPREHENSIVE ANALYSIS OF THE OBTAINED RESULTS AND PUBLISHED DATA

U okviru poglavlja Diskusija, dobijeni rezultati istraživanja su detaljno analizirani i razmatrani u kontekstu dostupnih literaturnih podataka.

Odavno je poznato da modeli hroničnog stresa, poput socijalne izolacije, ponavljane imobilizacije, maternalne deprivacije, hroničnog blagog stresa, kao i dugotrajne primene glukokortikoida, indukuju razvoj promena u ponašanju koje se mogu uporediti sa depresivnom simptomatologijom (Grippo i sar., 2008; Murray i sar., 2008; Donner i sar., 2012; Gong i sar., 2018; Shepard i sar., 2018). U datim modelima hroničnog stresa/depresije, u srvhu praćenja promena u ponašanju životinja, široku primenu ima FST, kao standardni skrining test. Rezultati velikog broja istraživanja su pokazali da promene koje se javljaju tokom FST i reflektuju depresivni fenotip obuhvataju: porast vremena imobilnosti i skraćenje vremena provedenog u plivanju i penjanju uz zidove cilindra (Detke i Lucki, 1996; Can i sar., 2012). U studiji Donner i sar. (2012), primenom neinvazivnog modela hiperkortizolemije, je pokazano da se kod odraslih mužjaka pacova nakon tretmana kortikosteronom (100 μg/mL ili 400 μg/mL, 21 dan) razvija depresivna simptomatologija, koji se manifestuje izraženim porastom perioda imobilnosti, kao i skraćenjem vremena provedenog u penjanju uz cilindar. Takođe, u modelu rane socijalne izolacije je zabeleženo, da se nakon 8 dana izolacije, javlja značajno povećanje vremena imobilnosti kod miševa (Gong i sar., 2018). Opisani rezultati su u skladu sa nalazima doktorske disertacije, koji ukazuju na razvoj depresivnog fenotipa nakon tronedeljnog ACTH tretmana adrenokortikotropnim hormonom, kao jednim od glavnih molekulskih medijatora stresa. Rezultati dobijeni u okviru ove disertacije, po prvi put upućuju na anksiolitički i antidepresivni efekat Mg u datom modelu depresivnog ponašanja, koji se manifestuje skraćenjem vremena imobilnosti i porastom vremena provedenog u plivanju, odnosno aktivnom ponašanju usmerenom ka traženju izlaza iz cilindra. Naime, tokom poslednje dve decenije, raste broj ispitivanja na temu homeostaze Mg i aktivnosti HPA ose. Interesantno je da restrikcija unosa Mg, putem hrane i deficit koji se potom javlja u organizmu, imaju prodepresivni i anksiogeni efekat kod glodara. Prve implikacije o

bihejvioralnim posledicama poremećaja homeostaze Mg u organizmu pojavile su se u istraživanju Singewald i sar. (2004) u kom je pokazano da se nakon 28 dana dijetetskog režima koji obezbeđuje 10% dnevnih potreba za Mg, kod životinja javljaju promene u ponašanju koje su analogne depresivnoj simptomatologiji i anksioznosti kod ljudi.

Pored bihejvioralnih promena, neophodno je pomenuti da su Sartori i saradnici (2012) u modelu deficijencije Mg kod miševa, zabeležili poremećaj aktivnosti HPA ose, koji se ogleda u porastu genske ekspresije CRF u hipotalamusu, kao i koncentracije ACTH u krvi (Sartori i sar., 2012). U skladu sa navedenim otkrićem su i nalazi dobijeni u okviru prve faze ove disertacije, koji ukazuju da primena Mg suprimira hiperaktivnost HPA ose indukovanu ACTH tretmanom, što za posledicu ima normalizaciju nivoa kortikosterona i pad koncentracije IL-6 u plazmi. U svetlu ovih otkrića, nekoliko autora razmišlja o uvođenju hiperaktivnosti HPA sistema kao potencijalnog biomarkera depresivnog poremećaja, što dodatno potvrđuje učestalost datog nalaza u animalnim modelima, ali i kod depresivnih osoba (Heuser i sar., 1994; Oosterhof i sar., 2016).

Kitamura i sar. (2002) su prvi razvili, a potom i implementirali ACTH model depresije, uz neznatne varijacije u protokolu. U pomenutom istraživanju, nakon dugotrajne primene ACTH (100 μg/dan, *s.c.*, 14 dana), kod mužjaka pacova Wistar soja, zapažen je izraženi porast kortikosterona u plazmi, koji reflektuje hiperaktivnost HPA osovine. Interesantno je da su davne 1999. godine Cratty i Birkle ukazali na inhibitorni *in vitro* efekat koji ispoljava magnezijum na oslobađanje CRF u ćelijskoj kulturi. Kasnije, u ispitivanju koje su sproveli Zhou i sar. (2018), primećeno je da se u modelu hroničnog blagog i nepredvidivog stresa, značajno povećava ekspresija, ali i aktivacija NMDA glutamatergičkih receptora, lokalizovanih u hipotalamusu, koji posreduju u oslobađaju CRF. Smatra se da porast signalizacije posredstvom NMDA receptora i neminovno sekrecije CRF, u znatnoj meri doprinosi porastu aktivnosti HPA osovine i hipersekreciji kortikosterona (Zhou i sar., 2018). Imajući na umu da Mg ispoljava aktivnost negativnog alosternog modulatora NMDA receptora i u skladu sa navedenim izveštajima, može se pretpostaviti da je u modelu depresije koji je implementiran u istraživanja u sklopu ove disertacije, Mg suplementacija suprimirala glutamatergičku transmisiju u hipotalamusu, što je za ishod imalo inhibiciju prekomernog oslobađanja kortikosterona i normalizaciju funkcije neuroendokrinog HPA sistema. Pored navedenih neuroendokrinih promena, izveštaji ukazuju da se kod osoba koje pate od depresija, u određenom broju slučajeva javlja i sistemska inflamacija, praćena porastom cirkulišućih IL-1, IL-2, IL-6, TNF-α, IL-10, IL-12, IL-13, kao i C-reaktivnog proteina (CRP,

eng. C-reactive protein) (Pace i sar., 2007; Nikkheslat i sar., 2015). Rezultati dobijeni u prvom setu eksperimenata, u okviru ove disertacije, ukazuju da hronična primena magnezijum sulfata dovodi do smanjenja nivoa IL-6 u plazmi zdravih, ali i životinja izloženih ACTH.

Prilikom ispitivanja neurohumoralnog odgovora na ACTH i Mg tretman, kao i na izlaganje akutnom stresu, u istraživanju u okviru ove disertacije, prvi put je pokazano da hronični tretman magnezijum sulfatom indukuje porast nivoa adrenalina, noradrenalina i serotonina kod zdravih mužjaka pacova, tokom izlaganja jedinke FST. Pored toga, primena magnezijuma je ostvarila protektivni efekat u modelu depresivnog ponašanja, što se, takođe, delom može objasniti porastom adrenalina i noradrenalina u plazmi tokom izvođenja FST. Pretpostavlja se da porast cirkulišućih kateholamina i serotonina zapravo određuje ponašanje jedinke i preusmerava odgovor na akutni stres ka aktivnostima koje imaju za cilj pronalaženje izlaza iz naizgled beznadežne situacije kojoj su podvrgnute u toku ovog bihejvioralnog testa. Nasuprot tome, izveštaji pojedinih ispitivanja ukazuju na inhibitorni efekat koji ispoljava Mg kada je u pitanju sekrecija kateholamina iz srži adrenalne žlezde kod mužjaka pacova (Shimosawa i sar., 2004; Komaki i sar., 2013). Opisane razlike najverovatnije proističu iz razlika u dužini tretmana, kao i činjenice da li su jedinke izložene akutnom stresu ili ne. Treba pomenuti da izveštaji ranijih kliničkih studija ukazuju da kod pacijenata koji boluju od depresivnog poremećaja nivo centralnih i perifernih kateholamina može biti povišen, snižen, ali i nepromenjen. Interesantno je, na primer, da akutna i hronična terapijska primena fluoksetina, uzrokuju primetan porast adrenalina i noradrenalina u plazmi, kod osoba koje pate od depresije (Blardi i sar., 2005).

Tokom izučavanja neurobioloških supstrata depresivnog poremećaja, nakon *post mortem* analize humanih uzoraka moždanog tkiva, zapaženo je da se kod pacijenata koji pate od depresije javlja atrofija prefrontalnog korteksa i hipokampusa i hipertrofija amigdala. Takođe, u velikom broju ispitivanja na humanim uzorcima, kao i u prekliničkim modelima, potvrđeno je da hroničan stres dovodi do supresije adultne neurogeneze u hipokampusu, i kod obolelih od depresije smanjeno je stvaranje novih neurona u hipokampusu u poređenju sa zdravim osobama (Duman i Monteggia, 2006). Rezultati dobijeni nakon imunohistohemijske analize markera proliferacije Ki-67 i neurotrofina BDNF u dentatnom girusu hipokampusa, ukazuju da hronična peroralna primena magnezijuma indukuje proliferaciju ćelija i porast nivoa BDNF proteina kod kontrolnih, nestresiranih životinja, ali i kod pacova izloženih dugotrajnom ACTH tretmanu, kao jednom od glavnih molekulskih medijatora stresa. Pomenuti rezultati istraživanja idu u prilog hipotezi da primena magnezijuma ima

antidepresivni efekat kod eksperimentalnih životinja koje ispoljavaju eminentne bihejvioralne i neuroendokrine promene, karakteristične za depresivni fenotip, rezistentan na terapiju tricikličnim antidepresivima. Prema dostupnim literaturnim podacima, ovo je prva *in vivo* studija u kojoj je pokazano stimulatorno dejstvo magnezijuma na proces adultne neurogeneze kod životinja. Interesantno je pomenuti da uprkos tome što stres i glukokortikoidi smanjuju proliferaciju i diferencijaciju neurona u hipokampusu, kao i ekspresiju BDNF, supresija procesa neurogeneze nije preduslov za ispoljavanje promena u ponašanju koje su analogne depresivnoj simptomatologiji. Takođe, u istraživanju u okviru ove disertacije, nije zabeležena smanjena neurogeneza kod životinja ACTH grupe. Međutim, ispitivanja su pokazala da je BDNF u hipokampusu, prevashodno u dentatnom girusu, neophodan za ispoljavanje antidepresivnog efekta (Adachi i sar., 2008).

U okviru prve faze istraživanja, uočeno je da hronično povišen nivo ACTH izaziva smanjenje telesne mase eksperimentalnih životinja, što je u skladu sa izveštajima drugih istraživanja. Naime, odavno je poznato da visok nivo cirkulišućih glukokortikoida dovodi do toga da životinje manje dobijaju na masi, ili ako su u pitanju veoma visoke doze glukokortikoida, javlja se progresivno smanjenje telesne mase (Donner i sar., 2012). Takođe, rezultati dobijeni u okviru prve faze disertacije su očekivano potvrdili ranije nalaze da hronična izloženost visokom nivou cirkulišućeg ACTH dovodi do hipertrofije nadbubrežne žlezde. Primena magnezijum sulfata, u implementiranom modelu depresivnog ponašanja, je delovala protektivno kod životinja ACTH/Mg grupe i antagonizovala je negativne efekte ACTH tretmana na telesnu masu, ali nije sprečila razvoj hipertrofije adrenalnih žlezda.

*In the **Discussion** section, the obtained results are analyzed in detail and overview of the available literature data is provided.*

Studies have shown that models of chronic stress, including social isolation, repeated immobilization, maternal deprivation, chronic mild stress model, as well as long-term glucocorticoid administration, produce behavioral changes in animals, indicative of depressive phenotype (Grippo et al., 2008; Murray et al., 2008; Donner et al., 2012; Gong et al., 2018; Shepard et al., 2018). FST has been for 40 years the most widely used screening method in assessing depressive-like behavior and new antidepressant drugs. A vast number of studies have shown that animals exposed to chronic stress or protracted glucocorticoid treatment display depressive-like behavior, manifesting in increased immobility time and decreased time spent swimming and/or struggling in the FST (Detke and Lucki, 1996; Can et al., 2012). In a non-invasive rat model for hypercortisolism, Donner et al. (2012) have

demonstrated that chronic corticosterone treatment (100 μg/mL and 400 μg/mL, 21 days) induce depressive-like behavior, manifesting in increased immobility time and significantly reduced time spent struggling. Furthermore, in a model of early life social isolation, after 8 days of isolation, immobility time is significantly elevated in animals (Gong i sar., 2018). The results obtained during first experimental phase of this dissertation, showing anxiety- and depressive-like behavior induced by ACTH, are in line with the abovementioned literature data. Furthermore, chronic Mg supplementation, in addition to the anxiolytic-like effects observed in the OFT, induced an antidepressant-like effect in this animal model of depression resistant to tricyclics, manifesting in reduced immobility time and increased time spent struggling. In the past two decades, numerous studies have investigated the relationship between Mg homeostasis and HPA axis activity. Evidence suggest that Mg-depletion leads to enhanced depression- and anxiety-related behavior in animals (Singewald et al., 2004). Namely, Singewald et al. (2004) have shown that compared to control mice fed with normal diet, mice receiving a low Mg diet (10% of daily requirement) displayed increased immobility time in the forced swim test, indicating enhanced depression-like behavior. Sartori et al. (2012) revealed that Mg-deficient diet in mice disrupts HPA axis activity, resulting in increase in preprocorticotropin- releasing hormone (prepro-CRH) mRNA expression in the hypothalamus, accompanied by elevated ACTH plasma levels. These data are in line with findings presented in this dissertation. Namely, 28-day-long Mg supplementation in rats attenuated HPA axis hyperactivity induced by ACTH, reflecting in a significant decrease in plasma corticosterone and IL-6 levels. This finding is particularly significant in terms of association of HPA axis hyperactivity and treatment resistance. HPA axis hyperactivity, one of the hallmarks of major depression, was confirmed in both preclinical and clinical research (Heuser et al., 1994; Oosterhof i sar., 2016). Results presented in this dissertation show that Mg supplementation reduced plasma ACTH and corticosterone levels, and this finding could, at least partly, be attributed to the previously shown inhibitory effect of Mg on glutamate mediated release of corticotrophin-releasing factor (Cratty and Birkle, 1999).

Kitamura et al. (2002) first developed and implemented ACTH model of depression, demonstrating that protracted ACTH treatment (100 μg/day, s.c., 14 days) evokes depressive-like behavior accompanied by HPA axis hyperactivity and elevated plasma corticosterone levels. In a model of chronic mild and unpredictable stress, Zhou et al. (2018) have shown a prominent increase in the expression levels, as well as activation, of hypothalamic N-methyl-Daspartate (NMDA) receptors, that mediate the release of CRF. Furthermore, it was

suggested that enhanced NMDA receptor signaling and consequently increased release of CRF, contribute to HPA axis hyperactivity. Numerous physiological roles of Mg have been described, including its activity as the negative allosteric modulator of receptors NMDA. Hence, it could be hypothesized, that in the present model of ACTH-induced depression, Mg decreased NMDA receptor signaling, resulting in reduced CRF release and supressed HPA axis activity.

Studies have demonstrated that some patients suffering from depression show signs and symptoms of systemic inflammation, accompanied by elevated levels of IL-1, IL-2, IL-6, TNF-α, IL-10, IL-12, IL-13 and C-reactive protein (CRP) (Pace et al., 2007; Nikkheslat et al., 2015). Results obtained in first experimental phase suggest that Mg reduces plasma IL-6 levels in control, as well as in rats exposed to protracted ACTH treatment. Furthermore, the present findings show that Mg induced a prominent increase in plasma adrenaline, noradrenaline and serotonine levels in male rats, during FST exposure. It has been proposed that this effect and increase in plasma adrenaline, noradrenaline and serotonine in response to acute stress, has a protective role and promotes active coping behavior. Some studies indicate that Mg inhibits adrenaline and noradrenaline release in male rats (Shimosawa et al., 2004; Komaki et al., 2013). However, these differences could be due to different experimental protocols, treatment duration and stress response. On the other hand, clinical studies have shown that in patients suffering from depression central and peripheral cathecholamine levels can be increased, decreased or unaltered. In addition, both acute and chronic fluoxetine treatment provoke an increase in plasma adrenaline and noradrenaline levels in depressed patients (Blardi et al., 2005).

In patients suffering from depression there are consistent reports on hippocampal and PFC volumetric decrease and amygdala hypertrophy. Molecular and cellular studies of stress, depression, and antidepressants have shown that stress is one of the most robust negative regulators of adult neurogenesis. (Duman i Monteggia, 2006). Results of the first phase of the dissertation have shown that chronic Mg supplementation enhances adult hippocampal neurogenesis in both controls and animals exposed to ACTH. Immunohistochemical analysis revealed that Mg provoked a significant increase in number of Ki-67 and BDNF immunopositive cells in the hippocampus. Importantly, the supportive role of Mg supplementation in enhanced neuroprotection and neurogenesis could contribute to its anti-depressive-like effect. In addition, in the present study, ACTH exposure showed no effects on hippocampal neurogenesis. Though, an abundance of evidence show that stress and

glucocorticoids decrease proliferation, neuronal differentiation and BDNF expression in adult hippocampus, several studies have demonstrated that impaired neurogenesis, per se, did not lead to depressive-like behavior in animals. However, studies have suggested that BDNF signaling mediates antidepressant effects (Adachi et al., 2008).

The results obtained in the first phase of dissertation have confirmed previous findings related to detrimental effects of stress and glucocorticoids on body weight. Elevated levels of plasma ACTH provoked a decrease in experimental animal's body weight, whereas Mg supplementation inhibited this effect. Donner at al. (2012) have shown that increased plasma glucocorticoid levels induce a decrease in body weight gain, or in case of very high glucocorticoid levels body weight loss. In addition, elevated ACTH levels plasma led to adrenal hypertrophy and Mg treatment could not reverse this effect.

U okviru druge eksperimentalne faze, uočeno je da Mg dovodi do primetnog porasta nivoa Cu, praćenog porastom odnosa Cu/Zn u prefrontalnom korteksu. Opisani efekat Mg, zajedno sa nalazima dobijenim u okviru prvog seta eksperimenata, o stimulatornom dejstvu Mg na oslobađanje adrenalina i noradrenalina, govori u prilog hipotezi o povezanosti između Mg i monoaminergičkog sistema. Sa druge strane, literaturni podaci ukazuju da deficit Cu kod glodara može dovesti do značajnog smanjenja nivoa noradrenalina u mozgu (Prohaska i Smith, 1982; Młyniec i sar., 2015). Rezultati ove disertacije upućuju na pretpostavku da smanjenjem nivoa Cu u prefrontalnom korteksu, ACTH izaziva poremećaj noradrenergičke transmisije, dok primena Mg inhibira ovaj efekat.

Povezanost između depresije i patohistoloških promena u miokardu je davno primećena, međutim, mehanizam nastanka i neurohumoralni medijatori, do danas, nisu u potpunosti razjašnjeni. Smatra se da je kod osoba koje boluju od depresije dvostruko do četvorostruko povišen rizik za nastanak kardiovaskularnih oboljenja. Rezultati dobijeni u drugom setu eksperimenata, u okviru izrade ove disertacije, su pokazali izraženi porast proliferacije fibroblasta i primetno povećanje sadržaja endomizijalnog kolagena nakon ACTH tretmana, što reflektuje prisustvo intersticijalne fibroze miokarda kod ovih životinja. Pored fibroze, zapaženo je da ACTH izaziva vaskularno remodelovanje, koje se ogleda u proliferaciji vaskularnih endotelnih ćelija u srcu i porastu ekspresije anti-apoptotskog markera Bcl-2 u ovim ćelijama. Prikazani rezultati su konzistentni sa izveštajima drugih istraživanja u kojima su opisane patološke promene koje se javljaju prilikom izlaganja hroničnom stresu ili visokom nivou cirkulišućih glukokortikoida. Omori i sar. (2014) su u modelu srčane

insuficijencije pokazali da kortikosteron stimuliše sintezu kolagena u fibroblastima miokarda, kao i da ovaj efekat nastupa zahvaljujući aktivaciji mineralokortikoidnih receptora u srcu. U skladu sa prikazanim rezultatima, su i nalazi studije Xinxing i sar. (2104), u kojoj je u modelu hroničnog blagog stresa, zabeleženo da se nakon 8 nedelja, kod životinja razvija depresivna simptomatologija, praćena patološkim promenama na miokardu, poput porasta nivoa apoptoze kardiomiocita, rupture miofibrila i unutrašnje membrane mitohondrija, kao i marginacije nuklearnog hromatina (Xinxing i sar., 2104). Pored toga, prilikom ispitivanja udruženih efekata psihosocijalnog stresa i gojaznosti, otkriveno je povećanje količine kolagena u intersticijumu, praćeno apoptozom kardiomiocita i remodelovanjem velikih koronarnih arteriola kod miševa (Agrimi i sar., 2019). Interesantno je da primena Mg u modelu depresije indukovane ACTH tretmanom, deluje kardioprotektivno, suprimirajući razvoj fibroze miokarda i vaskularno remodelovanje. Dobijeni nalazi u okviru druge faze izrade disertacije, su konzistentni sa rezultatima ispitivanja u kojima je pokazano da deficijencija Mg može dovesti do razvoja kardiomiopatije koju odlikuje prisustvo fokalne nekroze i fibroze miokarda (Bloom, 1988; Kumar i sar., 1997). Primećeno je da se kao rezultat deficijencije magnezijuma u organizmu javlja porast sinteze i deponovanja kolagana u miokardu eksperimentalnih životinja, kao jedan od parametara reparativne fibroze.

Interesantan je podatak da su i ACTH, kao jedan od glavnih medijatora stresa i Mg stimulisali proliferaciju kardiomiocita kod mužjaka Wistar pacova. Pokazano je da se u humanom srcu odraslih jedinki javlja proliferacija kardiomiocita nakon infarkta miokarda (Beltrami i sar., 2001) i kod pacijenata u terminalnoj fazi srčane insuficijencije (Kajstura i sar., 1998). Prirodni regenerativni kapacitet humanog miokarda u adultnom dobu je veoma mali, nedovoljan da spreči progresivni gubitak funkcije nakon oštećenja srčanog mišićnog tkiva i stoga nema klinički značaj. Identifikacija endogenih molekulskih medijatora i signalnih puteva koji stimulišu proliferaciju srčanih mišićnih ćelija i reparaciju miokarda, je od centralnog značaja za razvoj novih terapijskih strategija i povećanje preživljavanja i poboljšanje kvaliteta života kardiovaskularnih pacijenata. U drugom setu eksperimenata u sklopu ove disertacije, takođe je pokazano da hronična aplikacija ACTH, kao i peroralna Mg suplementacija dovode do značajnog smanjenja dijametra kardiomiocita. Interesantno je da, nasuprot očekivanjima, efekat magnezijuma na dati parametar je izraženiji, dok je kombinovana primena ACTH i Mg za posledicu imala normalizaciju prosečnog dijametra kardiomiocita. Molekulski mehanizmi i signalni putevi koji su u osnovi efekata ACTH i Mg na proliferaciju i dijametar kardiomiocita do danas nisu razjašnjeni. Pretpostavka je da opisani efekat na dijametar nastaje zahvaljujući

različitim fazama ćelijskog ciklusa u kojima su bili kardiomiociti u trenutku prikupljanja uzoraka. Drugo objašnjenje, koje ne isključuje prethodno, podrazumeva da su razlike u transnuklearnom dijametru kardiomiocita posledica ekscentrične hipertrofije koja se javila kod mužjaka pacova hronično izloženih ACTH.

The results obtained in the second phase of dissertation have shown that Mg treatment induced increase in the levels of Cu, accompanied by increased Cu/Zn ratio in the PFC, one of the key brain regions that shows functional alterations in depression. Previous studies have demonstrated that Cu deficit in the rodents may lead to the significant decrease in noradrenaline level in the brain (Prohaska and Smith, 1982; Młyniec et al., 2015). Present findings indicate that, by decreasing Cu content in the PFC, ACTH may lead to an impaired noradrenergic transmission, whereas chronic Mg administration in ACTH/Mg animals can reverse this effect. On the other hand, neither long-term Mg nor ACTH treatment in male rats exerted effects on Zn and Fe homeostasis in the PFC.

In the past decade, a link between depression and heart disease has gained attention, however underlying mechanisms and molecular mediators remain unknown. People who suffer from depression show a two-fold to four-fold increased risk of cardiovascular disease. The results presented in the second phase show that long-term administration of ACTH in male rats led to fibroblast proliferation and profoundly increased collagen deposition in the heart, indicative of cardiac fibrosis. Furthermore, ACTH induced vascular remodeling, reflecting in increased expression of proliferation marker Ki-67 and anti-apoptotic protein Bcl-2 in cardiac vascular endothelial cells. These results align with the literature evidence showing impaired cardiac morphology and functions induced by chronic stress and elevated levels of glucocorticoids. In a preclinical model of heart failure, Omori et al. (2014) demonstrated that in the presence of oxidative stress, corticosterone evoked cardiac fibrosis through mineralocorticoid receptors, in rats. Similarly, in a rat model of chronic unpredictable stress, Xinxing and colleagues (2014) showed detrimental cardiac aberrations, including disruption of myofibrils, interruption of inner mitochondrial membranes in cardiomyocytes, and increased cardiomyocyte apoptosis. Consistent with the findings presented in this dissertation, a recent study showed that chronic psychological stress in obese mice evokes interstitial fibrosis and vascular remodeling. Furthermore, this study revealed that psychosocial stress and obesity act synergistically and result in deterioration in cardiac function, reflecting in prominent decrease of ejection fraction (*Agrimi et al., 2019). Importantly, in the present model of depressive-like behavior, Mg application suppressed ACTH-evoked cardiac fibrosis and*

vascular remodeling in the heart. Literature data suggests that Mg deficiency produces a cardiomyopathy, harboring focal myocardial necrosis and fibrosis, and these observations further support the present findings. Mg deficiency in animals leads to increase in collagen synthesis and deposition in the heart (Bloom, 1988; Kumar et al., 1997.) Importantly, present findings indicate that both ACTH and Mg treatment promoted cardiomyocyte proliferation in rats. Beltrami et al. (2001) provided evidence of cardiomyocyte proliferation in the human heart following myocardial infarction, whereas Kajstura et al. (1998) observed this phenomenon in the end-stage of heart failure. However, regenerative capacity of human heart is very low and unable to prevent progressive loss of cardiomyocytes following MI and development of heart failure. Currently, treatment options are scarce and even incremental progress in therapeutic strategies directed toward attenuating cardiac remodeling could result in a reduced risk for the development of heart failure and increased survival of cardiovascular patients.

The results obtained in the second phase indicate that chronic ACTH application, as well as Mg exposure, induced a significant decrease in cardiomyocyte diameter. Surprisingly, Mg had a more profound impact on this parameter, whereas combined ACTH and Mg treatment unexpectedly resulted in the normalization of mean cardiomyocyte diameter. These differences in cardiomyocyte width might be due to different phases of the cell cycle at the time of tissue collection, or perhaps eccentric hypertrophy following ACTH treatment, manifesting in myofilament rearrangement and changes in protein synthesis.

Odavno je poznata uloga glukokortikoida i mineralokortikoidnih receptora u remodelovanju srca i razvoju srčane insuficijencije, međutim, do danas nisu sprovedena istraživanja u cilju ispitivanja direktnih efekata ACTH na srčane mišićne ćelije. Primena *in vitro* modela danas ima esencijalni značaj u izučavanju patoloških mehanizama i razvoju novih terapijskih pristupa (Gintant i sar., 2016). Kardiomiociti formirani indukcijom humanih pluripotentnih stem ćelija predstavljaju relativno novu eksperimentalnu platformu koja otvara dodatne mogućnosti, i istovremeno verno oslikava morfološke i funkcionalne karakteristike humanog srca. U trećoj fazi istraživanja u sklopu ove disertacije, implementiran je 3D model sferoida kardiomiocita nastalih indukcijom humanih pluripotentnih stem ćelija, i potom je uočeno da tretman suprafiziološkim koncentracijama ACTH indukuje porast nivoa ATP u kulturi. Izneta je pretpostavka da zabeleženo povećanje nivoa ATP u sferoidima kardiomiocita, u odsustvu proliferacije, ukazuje na povećan volumen – hipertrofiju kardiomiocita u kulturi. U prilog

datoj hipotezi govore rezultati prospektivne studije, u kojoj je tokom primene ACTH u terapiji miokloničnih napada kod pedijatrijske populacije, ehokardiografijom ustanovljeno prisustvo hipertrofije miokarda. Hipertrofija se razvila svega nekoliko dana od početka terapije, međutim, u roku od 5 meseci, od obustave primene ACTH, došlo je do normalizacije veličine i geometrije srca (Lang i sar., 1984; Bobele i sar., 1993).

The role of glucocorticoids and mineralocorticoid receptors in cardiac remodeling and development of heart failure has long been established, however, experiments performed in the third phase, for the first time aim to assess potential direct in vitro effects of ACTH on human cardiomyocytes. Cardiomyocytes derived from human stem cells represent a useful complement to animal experiments for studying cardiopathological mechanisms and signaling pathways. Furthermore, they are routinely employed in drug development for cardiotoxic liability testing of novel drug candidates (Gintant et al., 2016). However, conventional 2D monolayer cultures have numerous limitations, including lack of sensitivity and accuracy in predicting toxicity, and in the past decade focus has been shifted to new emerging 3D in vitro models, which more faithfully resemble human cardiac phenotypes and functions. In the third experimental phase, a 3D model of human cardiomyocyte spheroids was implemented and the results showed that exposure to very high ACTH concentrations produced an increase in ATP levels, which in the absence of proliferation could be due to increases in cell size. Therefore, it is plausible that exposure to high ACTH concentrations induced cardiomyocyte hypertrophy in vitro. Consistent with this finding, in prospectively studied pediatric patients with clonic seizures who received ACTH, significant myocardial hypertrophy was observed by echocardiographic evaluation few days after commencing therapy. However, after cessation of ACTH treatment, in the next 5 months, size and cardiac architecture were returned to normal (Lang et al., 1984; Bobele et al., 1993).

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D. OBJAVLJENI I SAOPŠTENI REZULTATI KOJI ČINE SASTAVNI DEO DOKTORSKE DISERTACIJE/ *RESULTS WHICH ARE PART OF THE DOCTORAL DISSERTATION*

Radovi objavljeni u naučnim časopisima međunarodnog značaja/ *Papers published in the international scientific journals*

1. **Petrović J**, Stanić D, Bulat Z, Puškaš N, Labudović-Borović M, Batinić B, Mirković D, Ignjatović S, Pešić V. 2018. Acth-induced model of depression resistant to tricyclic antidepressants: Neuroendocrine and behavioral changes and influence of long-term magnesium administration. *Hormones and Behavior* 105, 1-10. doi: 10.1016/j.yhbeh.2018.07.003.

Naziv časopisa/ *Journal name*: Hormones and Behavior
Impakt faktor (2020)/ *Impact factor (2020)*: 4,304
Kategorija/ *Category*: M21
Rang časopisa u oblasti *Behavioral Sciences/ Journal ranking in the field of Behavioral Sciences:* 10/53

2. **Petrović J**, Labudović-Borović M, Vorrink SU, Lauschke VM, Pejušković B, Pešić V. 2021. *Magnesium Research* pages 1-11. doi: 10.1684/mrh.2021.0484

Naziv časopisa/ Journal name: Magnesium Research
Impakt faktor (2020)/ *Impact factor (2020)*: 1,767
Kategorija/ *Category*: M23
Rang časopisa u oblasti Biochemistry & Molecular Biology/ *Journal ranking in the field of Biochemistry & Molecular Biology*: 265/297

Saopštenje sa međunarodnog skupa štampano u izvodu (M34)/ *Abstracts from the international scientific meetings*

- **Petrović J**, Stanić D, Mirković D, Batinić B, Plećaš B, Ignjatović S, Pešić V. 2016. Effects of long-term magnesium administration on levels of stress hormones and interleukin-6 after acute stress in rats chronically treated with adrenocorticotropic hormone. European Neuropsychopharmacology 26 (Suppl 2): S207.

E. ZAKLJUČAK - OBRAZLOŽENJE NAUČNOG DOPRINOSA DOKTORSKE DISERTACIJE/ *CONCLUSION – JUSTIFICATION OF SCIENTIFIC CONTRIBUTION OF THE DOCTORAL DISSERTATION*

Doktorska disertacija kandidata magistra farmacije Jelene Petrović se zasniva na ispitivanju uticaja hronične primene magnezijuma na ponašanje, parametre aktivnosti neuroendokrinog HPA sistema, adultnu neurogenezu u hipokampusu, kao i istraživanju kardioprotektivnih efekata, u modelu depresivnog ponašanja indukovanog ponavljanom primenom ACTH kod mužjaka Wistar pacova. Pored toga, istraživanja su obuhvatila i određivanje potencijalnih *in vitro* efekata ACTH na humane kardiomiocite u 3D modelu sferoida.

Detaljnom analizom priložene doktorske disertacije Komisija je konstatovala da je disertacija prikazana na jasan i pregledan način i da su svi postavljeni ciljevi u potpunosti realizovani. Eksperimenti su organizovani i sprovedeni u skladu sa savremenim standardima istraživanja u oblasti neuronauka i fiziologije kardiovaskularnog sistema. Na kraju doktorske disertacije prikazani su zaključci izvedeni na osnovu dobijenih rezultata i dostupnih literaturnih podataka. Podaci predstavljeni u disertaciji daju originalan doprinos boljem razumevanju sagledavanog problema. Svemu navedenom u prilog ide činjenica da su rezultati ove doktorske disertacije do sada publikovani u okviru dva rada u međunarodnim časopisima: kategorije M21 i kategorije M23.

Doctoral dissertation of the candidate Jelena Petrović, master of pharmacy, is based on the investigation of the effects of chronic Mg supplementation on behavior, parameters of the HPA axis activity and adult hippocampal neurogenesis. Furthermore, the focus is also on revealing potential cardioprotective effects of Mg in the model of depressive behavior, induced by chronic ACTH application and resistant to tricyclis. Lastly, potential direct in vitro effects of ACTH on cardiomyocytes were examined in a 3D spheroid model.

After detailed review of submitted doctoral dissertation, Committee ascertains that dissertation is presented in clear and concise manner and that all defined aims of doctoral dissertation are fully accomplished. All experiments were organized and conducted in accordance with contemporary standards in the research field, which enabled obtaining results that sustained achievement of predefined aims of the dissertation. At the end of the doctoral dissertation, the conclusions were made according too btained results, as well as, findings available in the literature. Findings presented in the dissertation provide a genuine contribution to research efforts in the explored scientific field. All above mentioned is supported by the fact that up to now the results of this doctoral dissertation are published in two papers in international journals category M21 and M23.

Na osnovu rezultata istraživanja u sklopu doktorske disertacije, mogu se izneti sledeći zaključci:

- U modelu depresije rezistentne na triciklične antidepresive, hronična primena magnezijuma ispoljava anksiolitički i antidepresivni efekat kod mužjaka pacova
- Hronični tretman magnezijumom deluje stimulatorno na proces adultne neurogeneze kod zdravih životinja, ali i u modelu depresije rezistentne na triciklične antidepresive. Opisani efekat se manifestuje značajnim porastom broja ćelija koje eksprimiraju marker proliferacije Ki-67 i neurotrofin BDNF u dentatnom girusu hipokampusa
- Hronična primena magnezijuma deluje modulatorno na aktivnost monoaminergičkog sistema, što se ogleda u porastu nivoa kateholamina i serotonina u plazmi, ali i nivoa Cu u prefrontalnom korteksu
- U modelu depresivnog ponašanja, rezistentnog na triciklične antidepresive, hronična primena magnezijuma suprimira hiperaktivnost HPA osovine. Navedeni efekat se manifestuje smanjenjem nivoa glavnog hormona stresa - kortikosterona u plazmi, i padom nivoa cirkulišućeg proinflamatornog citokina IL-6, koji je blisko povezan sa funkcijom HPA ose
- Hronični tretman magnezijumom suprimira negativne efekte ACTH na telesnu masu životinja i sprečava smanjenje telesne mase, koje se javlja prilikom dugotrajnog izlaganja ACTH. U modelu depresivnog ponašanja, visok nivo cirkulišućeg ACTH dovodi do hipertrofije nadbubrežne žlezde, dok protektivni efekat magnezijuma na ove endokrine žlezde izostaje.

- U modelu depresije rezistentne na triciklične antidepresive, hronična primena magnezijuma suprimira razvoj intersticijalne fibroze miokarda indukovane adrenokortikotropnim hormonom. Visok nivo cirkulišućeg ACTH, tokom dužeg vremenskog perioda, stimuliše proliferaciju fibroblasta i deponovanje endomizijalnog kolagena, parametara indikativnih za prisustvo fibroze u srcu eksperimentalnih životinja, dok magnezijum deluje kardioprotektivno i inhibira navedene efekte.
- U modelu depresivnog ponašanja, visoka koncentracija ACTH izaziva vaskularno remodelovanje, koje se ogleda u porastu broja endotelnih ćelija mikrocirkulacije srca koje eksprimiraju marker proliferacije Ki-67 i anti-apoptotski protein Bcl-2. Tretman magnezijumom u trajanju od 28 dana deluje protektivno i inhibira razvoj vaskularnog remodelovanja.
- Hronična primena magnezijuma kod zdravih životinja i u modelu depresivnog ponašanja, ali i dugotrajni ACTH tretman, stimulišu proliferaciju kardiomiocita, koja se manifestuje izraženim porastom broja srčanih mišićnih ćelija koje eksprimiraju Ki-67. Kod eksperimentalnih životinja i ACTH i Mg dovode do smanjenja dijametra kardiomiocita, dok je kombinovani tretman praćen normalizacijom ovog parametra.
- U trodimenzionalnom *in vitro* modelu sferoida kardiomiocita, dugotrajni tretman suprafiziološkom koncentracijom ACTH izaziva izraženi porast nivoa ATP, dok je ekspresija Ki-67 nepromenjena. Visok nivo ATP u kulturi, u odsustvu proliferacije, upućuje na hipertrofiju kardiomiocita izazvanu veoma visokim koncentracijama ACTH.

Na osnovu prikazanih rezultata istraživanja u okviru doktorske disertacije, može se zaključiti da je opravdano sprovesti kliničko ispitivanje i utvrditi ulogu Mg suplementacije, kao adjuvantnog terapijskog sredstva, kod osoba koje boluju od depresivnog poremećaja praćenog visokim nivoom cirkulišućeg kortizola.

Based on the presented results, as well as the discussion of doctoral dissertation, the following conclusions have been made:

- *Chronic Mg supplementation exerted anxiolytic and antidepressant effects in male Wistar rats in a model of depression resistant to tricyclics*
- *Chronic Mg application enhanced adult hippocampal neurogenesis in both healthy and animals exposed to ACTH treatment, manifesting in increased expression of proliferation marker Ki-67 and BDNF in the hippocampal dentate gyrus*

- *Chronic Mg treatment modulated the activity of monoaminergic system, resulting in increase of plasma catecholamine and serotonin levels and Cu concentration in the PFC*
- *In a model of depressive-like behavior resistant to tricyclics, chronic Mg supplementation suppressed HPA axis hyperactivity, resulting in decrease in plasma corticosterone and IL-6 levels*
- *Long-term Mg treatment suppressed detrimental effects of ACTH on body weight and prevented ACTH-induced loss of the body weight. ACTH provoked adrenal hypertrophy and this effect was not rescued by Mg*
- *In a model of depressive-like behavior resistant to tricyclics, chronic Mg treatment suppressed ACTH-induced cardiac fibrosis. ACTH treatment evoked proliferation of cardiac fibroblasts and endomysial collagen deposition, indicative of cardiac fibrosis, whereas Mg showed cardioprotective effects, preventing these changes*
- *In a model of depressive-like behavior resistant to tricyclics, elevated levels of ACTH induced vascular remodeling, reflecting in increased expression of Ki-67 and Bcl-2 in cardiac vascular endothelial cells, whereas long-term Mg application prevented this effect*
- *Both ACTH and Mg promoted cardiomyocyte proliferation, reflecting in increased expression of Ki-67 in cardiomyocytes. In addition, both ACTH and Mg led to decrease in cardiomyocyte diameter, whereas in ACTH/Mg group cardiomyocyte width was normalized*
- *In a 3D model of cardiomyocyte spheroids, high concentration of ACTH evoked an increase in ATP levels, whereas proliferation marker Ki-67 remained low, suggesting that ACTH induced cardiomyocyte hypertrophy in vitro.*

Taken together, these findings support further exploration of the cardioprotective effects of Mg supplementation and support its assessment in randomized, dietary trials in patients suffering from depressive disorder, harboring HPA axis hyperactivity.

F. PROVERA ORIGINALNOSTI DOKTORSKE DISERTACIJE/ ASSESSMENT OF ORIGINALITY OF DOCTORAL DISSERTATION

Korišćenjem programa iThenticate u Univerzitetskoj biblioteci Svetozar Marković, Beograd izvršena je provera originalnosti doktorske disertacije. Dobijena vrednost za *Similarity index* iznosi 10% i ovaj stepen podudaranja posledica je podudarnosti ličnih imena, citata, reči korišćenih u opisu metodologije, naziva terapijske grupe lekova, uobičajenih fraza koje se koriste u opisu rezultata istraživanja i statističke obrade, kao i publikovanih rezultata doktorandovih istraživanja, koji su proistekli iz rezultata ove doktorske disertacije, što je u skladu sa članom 9. Pravilnika. Shodno tome, može se izvesti zaključak da je priložena doktorska disertacija kandidata mag. farm. Jelene Petrović originalno naučno delo.

The program iThenticate was used to assess the originality of doctoral dissertation in the University Library Svetozar Marković, in Belgrade. The assessment showed *Similarity index* of 10% and this degree of concordance is due to similarities in personal names in the reference list, references, use of general terms to describe metodology, results and statistical analysis (for example: In table..; Statistically significant…P values less than…), standard name of therapeutic class and published data that comprise the results reported in this dissertation by the candidate, which is in accordance with the paragraph 9. in the Rule book. Taken together, the doctoral dissertation of the candidate Jelena Petrović is an original scientific contribution.

G. MIŠLJENJE I PREDLOG KOMISIJE

Na osnovu svega izloženog, može se zaključiti da je kandidat ispunio postavljene ciljeve u doktorskoj disertaciji pod nazivom **" Uticaj magnezijuma na ponašanje, neuroendokrine i promene na miokardu uzrokovane hiperaktivnošću osovine hipotalamus-hipofiza-nadbubreg kod pacova"**, te predlažemo Nastavno-naučnom veću Farmaceutskog fakulteta da prihvati Izveštaj i omogući kandidatu mag. farm. Jeleni Petrović odbranu doktorske disertacije.

*Based on the above considerations, it can be concluded that candidate fulfilled defined aims of the doctoral dissertation entitled: "**Effects of magnesium on behavior, neuroendocrine and changes in myocardium induced by hyperactivity of the hypothalamic-pituitary-adrenal axis in rats"**, and therefore we advise the Academic Council of the Faculty of Pharmacy – University of Belgrade to accept this Report and permit the candidate, master of pharmacy, Jeleni Petrović, defence of this doctoral dissertation.*

Beograd/ *Belgrade*

8.07.2021.

Članovi komisije/ *Committee members*

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