Original article

THE INFLUENCE OF N$^{\omega}$-NITRO-L-ARGININE METHYL ESTER ON ARGININE AND POLYAMINE METABOLISM IN RAT'S BRAIN TISSUE DURING EXPOSITION TO MICROWAVE RADIATION

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Exposition to microwave radiation (MW) from mobile phones, satellite communications, radio relays, radars and microwave devices in medicine induce disturbances in different organ systems. It has been shown that MW from mobile phones induce increasing of oxidative stress and apoptosis of neurons with impairment of blood brain barrier, disturbances of memory and space orientation. Citrulline and nitric oxide -NO are products of L-arginine by NO sintase-NOS. L-ornithine and polyamines are products of L-arginine by arginase. Nω-nitro-L-arginine methyl ester (L-NAME) competitive inhibits NOS and exerts neuroprotective effects.

The aim of this investigation was to determine the arginase, PAO and DAO activity, concentration of citrulline, as well as the effects of L-NAME on arginine and polyamine metabolism in brain tissue of rats exposed to MW.

Four groups of Wistar rats were investigated during 60 days: I-control-sham exposed, II (L-NAME)-rats treated with L-NAME (5 mg/kg b.w. i.p.), III (MW)-rats exposed to MW (4 h/day), IV (MW + L-NAME). The source of MW was mobile test telephone.

Decreasing activity of arginase (0.19±0.04 vs. 0.25±0.05 mmol/mg prot; $p<0.01$) and increasing of citrulline concentration (10.34±0.49 vs. 7.83 ±0.41 mmol/mg prot; $p<0.001$) were registered in the brain of MW exposed rats compared to controls. In L-NAME group there was a decrease of citrulline level ($p<0.05$), and increase in arginase activity ($p<0.05$) compared to controls. In the brain of exposed rats, the activity of PAO was significantly increased, while the activity of DAO was significantly increased vs. controls (1.12±0.10 vs. 0.79±0.09 U/mg prot; $p<0.001$ and 0.51±0.06 vs. 0.65±0.06 U/mg prot; $p<0.05$, prospectively). In MW+L-NAME group we registered increasing of DAO activity (0.61±0.04 vs. 0.51±0.06 U/mg prot; $p<0.05$) in the brain tissue compared with MW group.

Having considered the obtained results, we concluded that L-NAME exerted neuroprotective effects by preventing polyamine and arginine metabolism disturbances in the rats' brain under exposition to MW. *Acta Medica Medianae 2007;46(3):5-11.*

***Key words**: L-NAME, microwave radiation, arginine, polyamine, brain*

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Introduction

Radiation presents energy transmision through space by electromagnetic wawes (EW) or atomic particles. Electromagnetic wawes are consisted of electric and magnetic field which are indipendent entity on low frequency range but in high frequency range they are combined in unique electomagnetic field (EMF). Earth and biological sistems EMF are in extremly low frequency range. Microwave radiation (MW) used in mobile telephony, sattelite communication, radio braodcasting, radars, microwave heat devices and medical diathermia are range of 300 MHz to 300 GHz. Nowdeys, there are more then 2.5 bilion users of mobile telephones (1).

Iniciation of signal transduction after MW interaction with celll surface has important role in apoptosis, promotion of tumor growth and pathophysiology of neurodegenerative disorders such as Parkinson's and Alzheimer diseases. Exposure to MW (10 and 20 W/cm^2) significantly increased expresion of apoptotic Bcl-2 proteins in rats' testicualr cells (2). Increased oxydative stress after after MW exposure coul be one of the important mechanisms of apoptosis (3).

It is showed that MW radiation induces disturbances in ornithine decarboxilase (ODC) enzymes activity and impaired polyamines metabolism registred in cell cultures after MW exposure. Multiple variation in ODC activity and polyamine

metabolism disturbances are dependent of applied MW frequency, exposure times and tissues Activity of ODC in muscules cells decreased within 3-4 hours after exposure to MW radiation (4), but some literature data showed the opposite results (5).

Nonthermal MW effects induces serious changes on DNA molecules and proteins in the brain of experimental animals. This effects comprises appereance of micronuclera cells in bone marow, conformational changes of proteins and increased oxydative stress in brain tissue (6). The effects of MW on molecular level induce numerous cellular disorders such as increased leaking of calcium iones in human neuroblastoma cell cultures, reduced secretion of melatonin or unbalanced secretion in dopamino-opioide system (7). Many literature data indiced that MW radiation induced DNA damage in brain cells and exerts prooxydative potential which can be modified by melatonin application (8). Lai and Singh (1995) published that MW radiation induced single or double strains DNA breaks in brain cells on dose dependent maner thus exerts cancerogenic potential (9).

L-arginine is esential amino acids and precursor for protein, urea and creatinine sinthesis. Nitric oxyde sinthetase (NOS) induced translation of arginine to citruline and nitric oxyde (NO). Except vacular endothelial cells and activated macrofages, NO is produced in central nervous system, thymus, liver, lung, kidneys testises and gastointestinal tract. NO is imortant factors in numerous cells function regulation such as vascular tonus, neurotransmisions and immunogenesis. L-arginin is converted into ornithine by arginase. Decarboxilation of ornithine by ODC results in putrescine sinthesis. On the other side, decarboxilation of arginine by arginine decarboxylase (ADC) results in agmatine generation. It is thought that this metabolic pathway is characteristics of plant metabolism, but nowdays ADC ativity is proved in different mamalian tisues. Agmatine is hidrolised by agmatinase into putrescine or oxydised by diamino oxydase (DAO) into gamma-gvanidino-butiraldechide, which is further converted into GABA (10).

Polyamines (spermine, spermidine and putrescine) are ubiqvitous aliphatic base widely presented in biological systems. The greatest concentration of polyamines is registred in tissues with active protein sysnthesis (thymus, liver, pancreas). It is well known that polyamines increasing stability od double DNA helix. In interaction with nucleid acids, polyamines are involved in many vital proceses of cells proliferation, diferentiation as well as regeneration and reparation proceses (11). Polyamines exerts antioxydative activity and react with reactive oxygen species. Polyamine accumulation in cell membranes indicates ther role in regulation of peremeability (12).

Initial and rate limited step in biosinthesis of polyamines in amalian is decarboxilation of Lornithine under ODC activity with putrescine forming which is precursor for further sinthesis of spermine and spermidine. Biosinthesis of spermine and spermidine is ireversibile reaction while interconversion of polyamines back into putrescine is done under activity of two enzymes: spermidine/spermine N1-acetyl transferase (SSAT) and polyamijne oxydase (PAO), with production of toxyc acetamidopropanal and H_2O_2. This two enzymes also catalised conversion of spermine into putrescine by analog reactions. Putrescine could undervent oxydative desamination by the enzime diamino oxydase (DAO), with production of gamma-aminobutiraldechide and GABA.

Nω -nitro-L-arginine metil ester (L-NAME) is nonselective competative inhibitor of nitric oxyde sinthetase (NOS), whith primary effect on constitutive forms of NOS (nNOS-exprimed in brain tissue and eNOS-exprimed in endothelial cells) (13). In vivo study showed that application of L-NAME induced partial but permanent inhibition of nNOS. It is also proved that appliaction of L-NAME showed neuroptoective effects and inhibit neuronal damage (14).

The aim

The aim of this investigation was to determine the arginase, PAO and DAO activity, concentration of citruline, as well as the effects of L-NAME on arginine and polyamine metabolism in brain tissu of rats exposed to MW.

Matherial and methods

Experiments were performed on 28 adult male Wistar Albino rats (8–10 weeks old, 150-200 mg), bred at the Vivarium of the Institute of Biomedical Research, Medical faculty, Nis, under conventional laboratory conditions. All animals were housed collectively in polycarbonate cages 30x40x40 cm (WxLxH) and given ad libitum access to standard laboratory food and water. The housing room was maintained at 24°C with 42±5% relative humidity and had a 12–12-h light–dark cycle (light on 06:00–18:00 h). Four groups of experimental animals (each consisted of 7 animals) are included in study.

I group (control)-sham exposed with daily intraperitoneal (i.p.) application of 1.0 ml (0.9% NaCl)

II group (L-NAME)-animals with daily appliaction (i.p.) of 5 mg/kg BW L-NAME during 60 days

III group (MW)-animals exposed to MW radiation (4h per day) during 60 days. All animals got 1.0 ml (0.9% NaCl) (i.p.) every day 30 min before exposure.

IV group (MW + L-NAME)- rats with daily i.p. application of L-NAME (5 mg/kg BW), 30 minutes before MW exposure from mobile phones (4 h per day) during 60 days.

Experimental model of MW exposure

The microwave radiation was produced by a mobile test phone (model NOKIA 3110; Nokia Mobile Phones Ltd.) connected to a Communication Test Set PCDK with PC and appropriate software module. During microwave exposure seven freely moving rats were kept in a pure (i.e. lacking any metallic fittings) plastic

cage. In the present study, an electromagnetic near-field signal for GSM (Global System for Mobile communication) at 900 MHz (magnetic field B=4.68 μT to 8.69 μT) with continuous wave, mobile phone system was used. Source of MW was situated in the plastix box in the center of cage (maximal distance from the floor was 3 cm and from the corners 28.2 cm). Electromagnetic fields parameters in cage were measured several times during experimental exposition. The whole-body specific energy absorption (SAR) rate was estimated as 0.025-0.05 W/kg using data for a rotating ellipsoidal model of a rats.

Seven animals from each group was sacrified after 60 days. Rats were anesthetized with ketamine HCl (2 ml/kg), administered intraperitoneally (i.p.), before sacrificing and after 15h fasting period.

Preparation for biochemical analysis

For biochemical analysis the brain tissue was cut in small pieces, washed in ice-cold isotonic NaCl solution and freezed on –20°C. Brain tissue was homogenized (20% homogenisate) in ice-cold destilated water.

Biochemical analysis

The activity of arginase in brain homogenate was estimated by Porembska and Kedra (1975) mehod. Enzyme activity are measured by released free ornithine concentration. This Chinard reaction is based on fact that ornithine in adition of concentrated acet acid react with ninhidrine and create coloured compound with maximum absorbance at 515 nm. Arginase activity was expressed as μmol/mg tissue protein

Citruline contrentration, was determined spectrophotometrically using diacetil monoxime reaction by Boyde (1980) method. Citruline concentration was expressed in μmol/mg of protein.

The activity of PAO and DAO were determined spectrophotometrically by Bachrach and Reches (1966) method which is based on measurements of created aminoaldechide. Spermine is used as a supstrate for PAO and putrescine for DAO. Activity of PAO and DAO were expresed as U/mg tissue protein

Determination of proteins. Brain proteins were determined according to Lowry's method (1951), using bovine serum albumin as standard.

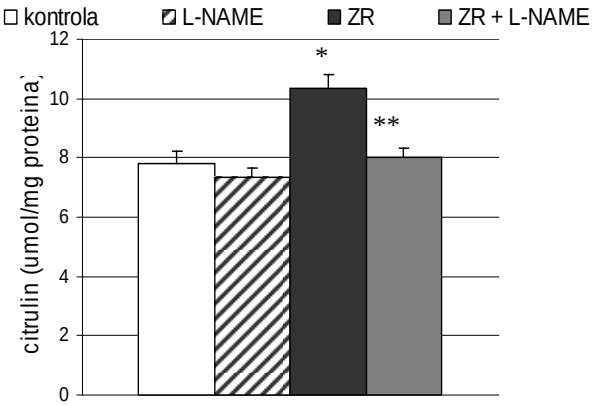
Statistical analysis

Data were analysed using a commercially available statistics software package (SPSS® for Windows, v. 9.0, Chicago, USA). Results were presented as means ±SD. Statistical significance was determined at level of $p<0.05$ using the Student's t-test.

Results

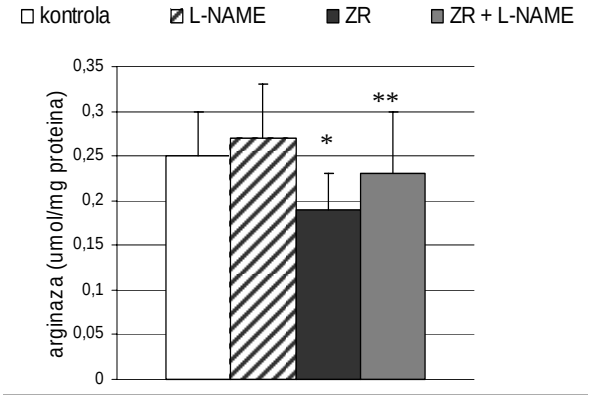
In the MWs group, 60 days of exposure to mobile phone produces a significant decrease in the brain tissue arginase activity (0,19±0,04 vs. 0,25±0,05 μmol/mg proteina; $p<0,01$) and increase of citruline concentration (10,34±0,49 vs. 7,83±0,41 μmol/mg proteina; $p<0,001$) when compared with control group.

Application of L-NAME to MW exposed animals (MWs+L-NAME group) significantly decreased citruline levels ($p<0,05$) and increased arginase activity ($p<0,05$) when compared with MWs groups (Graphic 1 and 2).



*p<0.001 (vs control);
**p<0.05 (vs radiation)

Graphic 1. Citriline concentration in brain of MW exposed animals

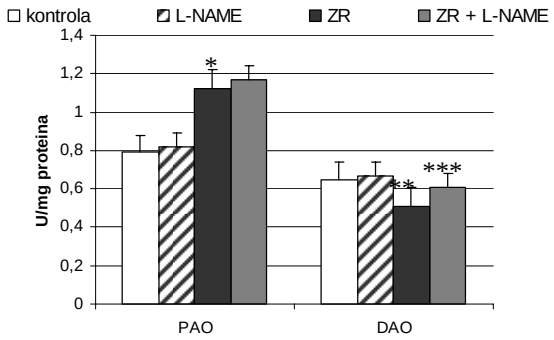


*p<0.01 (vs control);
**p<0.05 (vs radiation)

Graphic 2. Arginase activiry in brain of MW exposed animals

In the brain of MW's exposed animals PAO activity is significantly higher compared to control animals (1,12±0,10 vs. 0,79±0,09 U/mg proteins; $p<0,001$). Activity of DAO in the same tissue showed the opposite trend, and in MW's exposed animals DAO activity was significantly lower compared to control (0,51±0,06 vs. 0,65±0,06 U/mg proteins; $p<0,05$) (Graphic 3).

Intraperoteoneal application of L-NAME to animals exposed to MW (MW + L-NAME) induced moderate raise of PAO activity (1,17±0,07 vs. 1,12±0,10 U/mg proteins; p=ns.) and significant raise of DAO activity (0,61±0,04 vs. 0,51±0,06 U/mg proteins; $p<0,05$), compared to MW group (Graphic 3).



*p<0.001 (vs control);
**p<0.05 (vs control);
***p<0.05 (vs radiation)

Graphic 3. PAO and DAO activity in rats brain's tissue

Discusion

The dangers from electromagnetic field (EMF) exposure by using cellular phones are huge because their effects on human health and environment are not visible in short-time period. The growing concern about possible effects on the brain after microwave (MW) exposure from mobile phones, especially in children, have been discussed in many countries. One of the first observations about negative impact of MW radiation on human health indiced many nonspecific symptoms such as iritability, neurovegetative distonia and insomnia in workers on radar installation. It is showed that experimental animals exposed to MW expresed disorders of long term memory and orientation ability (15).

In normal conditions nitric oxyde sinthetase (NOS) and arginase are competative agonosts for L-arginine, converted them into citruline+NO or polyamines. It is confirm in the brain tissue of MW exposed rats where significant increasing of citruline concentration was followed by decreasing of arginase activity (Graphic 1 and 2). It is most likely that MW rerout L-arginine catabolism to citriline and NO sinthesis, but not to ornithyne (polyamines) sinthesis.

Ilhan et al. (2004) showed that exposure to mobile telephone MW during 7 days (1 h/per day) induced significant increasing of NO (16). Significant increasing of NO in rats' retinal and brain tissues were showed after 60 days of MW exposure (17). Ozguner et al. (2005) experimentaly proved significant increasing of NO in rats' myocardial cells after 10 days of mobile phones MW exposure (30 min/per day) (18).

Nitric oxyde is important modulator of many physiological proceses in CNS such as interneuronal communication, synaptic plasticity, releasing of neurotransmiters etc. (19). NO exerts many toxic prperties by forming nitoxil radicals, peroxinitrite or throught NMDA sistem. Increased neuronal sinthesis of NO contibute to cell death while increased endothelieal NO synthesis exerts neuroprotective role. NOS inhibitors which antagonise eNOS and nNOS are not effective because they compromited cerebral circulation (14).

Bauer et al. (2001) showed that NO directly inhibite enzime ornithine decarboxilase (ODC) by S-nitrosilation of sulfhidrile (SH) group in cistein (20). It could be assumed that increased NO and citruline production in brain of MW exposed rat, could lead to polyamine synthesis inhibition (directly by inhibition of ODC).

There are two isoforms of arginase enzime named arginase I and arginase II. Activity of citosolic arginase I in periportal hepatocites contibuting to urea forming, while activity of arginase II was detected in brain, kidneys and intestinum (21). It is hipothesed that arginase could regulated aviability of arginin for polyamines sinthesis, because arginase activity is coinduced with ODC and cells with arginase deficity could not proliferated in cullture without ornithine and polyamine suplementation. The great quantities of polyamines are presented in neuronal tissue (21).

It is ahowed that brain's cells are very sensitive on decreased polyamines concentration which is followed by compensative mechanism activation aimed to preserve normal levels of this compounds (12). In this study we registred decreasing of arginase activity and increasing of citruline concentration, which most likely indicate that decreased synthesis of brain's polyamines was occurred under MW exposure

There are many controversy about polyamines roles in brain's tissue. They have paradoxal role in apoptosis induction or it's prevention (22). Neuroprotective roles of polyamines proceed from their anti-apoptotic and anti-oxydative properties with influencing neuronal excitability and chromatine stabilisation (23). Spermin is stong inhibitor of increased mitochondrial membrane permeability thus stopping citochrome c leacking from mitochondria. and inhibiting apoptosis (24).

The mechanisms of neuronal damage dependent of polyamines are: 1) influx of Ca^{2+}, and neurotransmiters release in regions with increased putrescine production, 2) stimulation of NMDA receptors pathway, 3) releasing of citohrome c from mitochondria and activation of caspase-3 with induction of apoptosis by spermine (25).

Cerebelar ODC/polyamine system is very vulnerabile in patological conditions (including brain disorders after MW exposure). It is showed that changes in polyamine metabolism conected with CNS damage have important role in neuronal degenerations (26).

Significant increasing of PAO activity in rats' brain after MW exposure (Graphic 3) could be explained by intesified polyamines interconversion, because this enzymes catalised conversion of spermine into spermidine and spermidine into putrescine. This pathway enable regulation of plyamines levels and their disposition. During catalitic conversion spermine/spermidine into putrescine, under enzyme system SSAT/PAO, releasing of 3-acetaminopropanal and H_2O_2 is occured. Hidrogen peroxyde and aminoaldechides, produced during polyamines degradation have citotoxic proerties. Citotoxicity of 3-aminopropanal is regulated by acroleine forming. Toxic H_2O_2 could damaged proteins, DNA and

lipida and induced apoptosis. Many literature data indicated that polyamine toxicity are the result of H_2O_2 releasing during catabolisms (27). Some polyamines metabolic intermediers induced MDA forming (8,12).

Significant oxidative damage of DNA molecules and induction of apoptosis are registred during MW exposure probably caused by increased activity of spermine oxydase (SMO) and polyamine oksidase (PAO). Increased activity of these enzymes in brain cell nucleus and citoplasm induced increasing production of H_2O_2 and OH^-, with increased level of oxydative stress (28).

Agmatine is modulator of intracellular polyamines leves. It reduces spermine and spermidine concentration inducing activity of spermidine/ spermine N^1-acetil transferase (SSAT). It is showed that agmatine induses apoptosis, by reducing cell's polyamines levels. Cultured hepatocites supplied with agmatine showed increased number of apoptotic cells, due to increased agmatine degradation by DAO and releasing of toxic H_2O_2 (29). Galea i saradnici (1996) su dokazali da je agmatin kompetitivni inhibitor NOS, naročito njegove inducibilne izoforme (30).

Many patological conditions are associated with changing in DAO activity. Brain's tumours are associated with decreasing activity of DAO. Rats' brain showed significant increasing in DAO activity after 60 days MW exposure compared to control animals (Graphic 3). Decreased DAO activity in MW exposed brain's tissue probably decreasing putrescine catabolism with consequent increasing of putrescine concentration. Increasing of putrescine level in brain tissue exerts neuroprotective role.

The results showed in Graohic 1 and 2 indicated that aplication of L-NAME decreased citruline concentration in MW exposed rat (MW + L-NAME group) as well as increased arginase activity compared to MW exposed rats. ozračenim Having in the mind that L-NAME inhibits synthesis of NO and citruline from arginine, it is the most expected that appliaction of L-NAME would increased arginase activity. On that way, appliaction of L-NAME, would redirected catabolism of L-arginine toward poliamine synthesis but not toward citruline and NO production. We could hypothesis that appliacation of L-NAME prevent decreasing of polyamine levels in MW exposed brain.

Increased DAO activity was registred MW exposed in rats with i.p. applicaton of L-NAME, compared to MW exposed rats (Graphic 3). It could indicate on intensified putrescine catabolic proceses in mentioned conditions (31). Having in the mind that inhibitors of NO synthesis reduced GABA releasing in the brain cortex (32), it is posible that reduced NO levels after MW exposure, activated alternative pathway of GABA synthesis from putrescine catalised by DAO, which present logical explanantion for increased activity of this enzymes.

Conclusion

Obtained results indicate that in rats brain tissue occured significant increasing of citruline and PAO activity folowed by decreasing of arginase and DAO activity. after 60 days of MW exposure. Appliacation of L-NAME to MW exposed rats prevent disturbances in arginine metabolism by increased arginase and DAO activity, citruline and polyamines levels.

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UTICAJ N$^{\omega}$ -NITRO-L-ARGININ METIL ESTRA NA METABOLIZAM ARGININA I POLIAMINA U MOŽDANOM TKIVU PACOVA U TOKU IZLAGANJA MIKROTALASNOM ZRAČENJU

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Izlaganje mikrotalasnom zračenju (MW), koje se koristi za mobilnu telefoniju, satelitsku komunikaciju, radio emitere, radare, mikrotalasne toplotne uređaje i medicinsku dijatermiju dovodi do pojave poremećaja u različitim organskim sistemima. Dokazano je da MW mobilnih telefona dovodi do povećanja nivoa oksidativnog stresa i apoptoze neurona, narušavanja integriteta krvno-moždane barijere i poremećaja dugotrajne memorije i sposobnosti orijentacije. Iz L-arginina se pod dejstvom enzima azot monoksid sintaze (NOS) stvaraju citrulin i azot monoksid (NO), a pod dejstvom arginaze nastaju L-ornitin i poliamini (neophodni za rast, proliferaciju i regeneraciju ćelija). Nω-nitro-L-arginin metil estar (L-NAME) je neselektivni kompetitivni inhibitor NOS, koji pokazuje neuroprotektivno dejstvo i sprečava oštećenje neurona.

Cilj ovog istraživanja bio je da se nakon izlaganja pacova MW mobilnog telefona, u moždanom tkivu odredi: aktivnost arginaze, količina citrulina, katabolizam poliamina (određivanjem aktivnosti PAO i DAO), kao i efekat L-NAME na metabolizam arginina i poliamina.

Wister pacovi bili su podeljeni u četiri eksperimentalne grupe: I (kontrola), II (L-NAME) – životinjama je 60 dana svakodnevno davan L-NAME (5mg/kg TM), III (ZR) - životinje su 60 dana izlagane MW (4h/dnevno), IV (ZR + L-NAME) – pacovi kojima je aplikovan L-NAME, 60 dana (4h/dnevno) su izlagani MW mobilnog telefona.

U mozgu pacova koji su bili izloženi MW zabeleženo je sniženje aktivnosti arginaze (0.19±0.04 naspram 0.25±0.05 μmol/mg proteina; $p<0.01$) i značajno povišenje nivoa citrulina (10.34±0.49 naspram 7.83 ±0.41 μmol/mg proteina; $p<0.001$), u odnosu na kontrolne životinje. Aplikovanje L-NAME zračenim pacovima dovodi do sniženja nivoa

citrulina ($p<0.05$), kao i do povišenja aktivnosti arginaze ($p<0.05$), u odnosu na kontrolu. Aktivnost PAO u tkivu mozga ozračenih pacova je značajno povišena, dok je aktivnost DAO značajno snižena u odnosu na kontrolne životinje (1.12±0.10 naspram 0.79±0.09 U/mg proteina; $p<0.001$ i 0.51±0.06 naspram 0.65±0.06 U/mg proteina; $p<0.05$). Kod pacova koji su izlagani mikrotalasnom zračenju, a kojima je aplikovana L-NAME, zapaženo je povišenje aktivnosti DAO (0.61±0.04 naspram 0.51±0.06 U/mg proteina; $p<0.05$) u tkivu mozga u odnosu na ZR + L-NAME grupu.

Aplikovanjem L-NAME pacovima koji su izlagani MW dolazi do sprečavanja poremećaja metabolizma arginina i poliamina u moždanom tkivu i tako ova supstanca pokazuje neuroprotektivno dejstvo. *Acta Medica Medianae 2007;46(3): 5-11.*

Ključne reči: *L-NAME, mikrotalasno zračenje, arginin, poliamini, mozak*