sciendo

ISSN 1820-8665

Vol.19•No2 • JUNE 2018

Serbian Journal of Experimental and Clinical Research

2000

Serbian Journal of Experimental and Clinical Research

2000

Print
Faculty of Medical Sciences,
University of Kragujevac

Indexed in
EMBASE/Excerpta Medica, Index Copernicus, BioMedWorld, KoBSON, SCIndeks, Chemical Abstracts Service, Cabell's Directory, Celdes, CNKI Scholar (China National Knowledge Infrastructure), CNPIEC, EBSCO Discovery Service, Elsevier - SCOPUS, Google Scholar, J-Gate, Naviga (Softweco), Primo Central (ExLibris), ReadCube, SCImago (SJR), Summon (Serials Solutions/ProQuest), TDOne (TDNet), WorldCat (OCLC)

Address:
Serbian Journal of Experimental and Clinical Research, Faculty of Medical Sciences, University of Kragujevac
Svetozara Markovica 69, 34000 Kragujevac, PO Box 124
Serbia
http://www.medf.kg.ac.rs/sjecr/index.php

SJECR is a member of WAME and COPE. SJECR is published four times circulation 250 issues
The Journal is financially supported by Ministry for Science and Technological Development, Republic of Serbia
ISSN 1820 – 8665

Table Of Contents

CONSIDERATION OF THE THERAPEUTIC POTENTIAL OF IRRIGANTS IN ENDODONTIC THERAPY

Dajana Nogo-Zivanovic[1], Ljiljana Bjelovic[1], Vladimir Ivanovic[2], Tatjana Kanjevac[3], Irena Tanaskovic[4]
[1] Department for Restorative Dentistry and Endodontics, Faculty of Medicine, University of East Sarajevo, Foca, Bosnia and Herzegovina
[2] Department of Restorative Odontology and Endodontics, School of Dental Medicine, University of Belgrade, Belgrade, Serbia
[3] Department of Preventive and Pediatric Dentistry, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[4] Department of Histology and Embryology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia

RAZMATRANJE TERAPIJSKIH MOGUĆNOSTI IRIGANASA U ENDODONTSKOJ TERAPIJI

Dajana Nogo-Živanović[1], Ljiljana Bjelović[1], Vladimir Ivanović[2], Tatjana Kanjevac[3], Irena Tanasković[4]
[1] Katedra za restaurativnu stomatologiju i endodonciju, Medicinski fakultet, Univerzitet u Istočnom Sarajevu, Foča, Bosna i Hercegovina
[2] Katedra za restaurativnu odontologiju i endodonciju, Stomatološki fakultet, Univerzitet u Beogradu, Beograd, Srbija
[3] Katedra za preventivnu i dečiju stomatologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[4] Katedra za histologiju i embriologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija

Received / Primljen: 13. 10. 2016. Accepted / Prihvaćen: 13. 12. 2016.

ABSTRACT

The main objective of endodontic treatment is to remove vital and necrotic remnants of pulp tissue and microorganisms and their toxic products from the root canal. During chemo-mechanical endodontic preparation, a smear layer is formed on the wall of the canals. Due to an inability to remove all tissue remnants and the smear layer from the root canal by mechanical instrumentation, it is necessary to use irrigation to ensure sufficient cleaning and disinfection of the largest part of the root canalicular system. The most commonly used irrigants are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), citric acid and chlorhexidine (CHX). Recently, the irrigants QMix and MTAD have been introduced to the market. They are a mixture of different components having antimicrobial, organolytic and mineralytic effects on canal detritus and the smear layer. This review article investigates irrigants in terms of the nature of their effect, their efficiency, optimal concentration, and method of use, and the interactions between the irrigants most commonly used in endodontic therapy are discussed, with special emphasis on QMix and MTAD.

Keywords: *endodontic treatment, smear layer, endodontic irrigants*

SAŽETAK

Osnovni cilj endodontske terapije je uklanjanje vitalnih i nekrotičnih ostataka pulpnog tkiva, mikroorganizama i njihovih toksičnih produkata iz kanala korena zuba. U toku hemo-mehaničke obrade kanala korena na zidovima se formira razmazni sloj. Zbog nemogućnosti da se mehaničkom obradom uklone svi ostaci tkiva i razmazni sloj iz kanala korena, neophodno je koristiti irigaciju, kako bi se obezbedilo čišćenje i dezinfekcija najvećeg dela kanalikularnog sistema korena. Najčešće upotrebljivana sredstva za ispiranje kanala korena su natrijum-hipohlorit (NaOCl), etilendiaminotetraacetatna kiselina (EDTA), limunska kiselina i hlorheksidin (CHX). U novije vreme na tržištu su se pojavili irigansi, kao što su QMix i MTAD. Predstavljaju mešavinu različitih komponenti koje ispoljavaju antimikrobno dejstvo, kao i organo- i mineralolitički efekat na kanalni detritus i razmazni sloj. U ovom preglednom članku razmatran je način dejstva, efikasnost, optimalna koncentracija, način upotrebe i međusobna interakcija najčešće korišćenih iriganasa u endodontskoj terapiji, sa posebnim osvrtom na QMix i MTAD.

Ključne reči: *endodontski tretman, razmazni sloj, endodontski irigansi*

INTRODUCTION

The most common aetiological factors causing pulp and periapical diseases are microorganisms. The main objective of endodontic treatment is to remove vital and necrotic remnants of the pulp tissue, microorganisms and their toxic products from the root canal. Due to the anatomical complexity of the root canal system and the presence of numerous isthmi and intercanal communications and pulp-periodontal communications, a significant part of the intracanal area remains inaccessible to the mechanical effects of endodontic instruments. The research of Peters et al. shows that regardless of the technique of preparation, approximately 35% of the canal surface remains mechanically uninstrumented (1).

During chemo-mechanical preparation of the root canal, a smear layer 1-2 microns thick is produced on the walls. This layer contains remnants of vital and/or necrotic

sciendo
UDK: 616.314.16/.18-085 / Ser J Exp Clin Res 2018; 19 (2): 103-112
DOI: 10.1515/SJECR-2016-0095

Corresponding author: Tatjana Kanjevac, DMD, PhD;
Department for Preventive and Pediatric Dentistry, Faculty of Medical Sciences, University of Kragujevac, Serbia;
Svetozara Markovica 69, 34000 Kragujevac, Serbia; Tel +38134306800, E-mail: tatjanakanjevac@yahoo.com

pulp tissue, micro-organisms and their toxins and dentin particles of different size (2). The presence of a smear layer on the canal walls may partially or totally block the dentinal tubules and prevent the effects of irrigants and intracanal medicaments, obstruct the adhesion of materials for definitive obturation and provide a potential route for micro leakage (2). It also presents a nutritious foundation for the growth and multiplication of microorganisms. All of these factors lead to the failure of endodontic therapy, which is the reason why the removal of the smear layer is recommended (2).

Due to an inability to completely remove the remains of the residual tissue and smear layer from the root canal by mechanical instrumentation, it is necessary to use irrigation, which ensures cleaning and disinfection of the largest part of the canalicular system (3, 4). An ideal irrigant should possess antibacterial and fungicidal effects; not irritate periapical tissue; be chemically stable; possess prolonged antimicrobial activity; be effective in the presence of blood, serum and protein derivatives from the tissue; remove completely the smear layer; possess a low surface tension and the ability to penetrate into the dentinal tubules and disinfect them; not interfere with reparative processes in the periodontal tissue; not overpaint the tooth; not have antigenic, toxic or carcinogenic effects on surrounding vital structures; not have a negative effect on the physical and chemical properties of the dentin; not interfere with the adhesion of materials for the definitive obturation, and be easily prepared and applied to the prepared canal (5).

Currently, there is no single irrigant that meets all of the previously mentioned requirements, so that in everyday clinical practice it is necessary to combine irrigants. The most commonly used irrigants for rinsing the root canal are sodium hypochlorite (NaOCl), ethylenediaminetetraacetic acid (EDTA), citric acid and chlorhexidine (CHX) (6), while recently, irrigants such as QMix and MTAD have been introduced to the market. Both are a mixture of different components designed to dissolve organic and mineral parts of the canal detritus and smear layer, as well as to have antimicrobial effects. The use of MTAD and QMix as final irrigants should simplify the procedure of irrigation, prevent interaction between different irrigants and should not be detrimental to the mechanical, physical and chemical properties of the root canal dentin.

Development of new irrigants, and their use in an appropriate manner, is imperative for successful endodontic treatment. Thanks to the existence of a number of research methods, such as electron microscopy (7, 8, 9, 10), in tests of cytotoxicity (11, 10, 12) and antibacterial efficacy (13, 14, 15, 16, 17, 18), today it is possible to evaluate the efficiency of irrigants used in endodontic therapy in a relatively simple way.

This review article presents a brief overview of current knowledge of the effect, efficiency, optimal concentration, method of use and interaction of the most commonly used irrigants in endodontic therapy, with special emphasis on QMix and MTAD.

SODIUM HYPOCHLORITE (NAOCL)

Sodium hypochlorite is a solution originally used for bleaching and disinfection (19). Due to its strong antimicrobial effect and its ability to dissolve organic tissue, it is the irrigant that is most often used during endodontic therapy (6, 20, 21). Due to its effect on soft tissue, NaOCl should be used cautiously, without the risk of transferring the irrigant over the apex. In the case of transfer of the solution onto the periapical tissue, pain can occur, as well as oedema, bleeding and even paraesthesia (22, 23). The chemical reaction of NaOCl with organic tissue proceeds through three phases. First, saponification of NaOCl dissolves the fatty acids by converting them into glycerol and fatty acid salts. This is followed by neutralization of amino acids by NaOCl caused by the formation of water and salt, with the release of hydroxyl ions, which strongly reduces the pH. Finally, the chlorination reaction, when hypochlorous acid from NaOCl comes into contact with organic tissue, acting as a solvent, leads to the release of chlorine, which reacts with the amino groups of proteins to form chloramine, which interferes with cell metabolism (24). The antibacterial effect of chloramine is based on its inhibitory effect on bacterial enzymes, leading to oxidation of sulphydryl groups (SH). Hypochlorous acid (HOCl-) and hypochlorite ions (OCl-) cause the degradation of amino acids and protein hydrolysis (24). NaOCl is a strong base with a pH of 11, which is the basis of its antimicrobial effect, and its mode of action is similar to that of calcium hydroxide.

For endodontic treatment NaOCl is used at concentrations ranging from 0.5% to 6%. In a wide search of the literature, no clear recommendation was found for the best concentration to be used in endodontic therapy. Higher concentrations of NaOCl dissolve organic tissue better (25), but they have a stronger toxic effect (26). The toxicity of this solution can be overcome by using a lower concentration with prolonged periods of irrigation, and by using larger amounts of irrigants, by which the same antimicrobial efficacy and effect on dissolving organic tissue are achieved as at high concentrations (24, 26-30). The effect of NaOCl may be enhanced by increasing the concentration, heating, using a prolonged period of irrigation and sonic or ultrasonic activation (27).

In the presence of soft and dentin tissue, chlorine is released, but a weakening of the effect of NaOCl occurs (31-33). Therefore, a continuous renewal of the solution is necessary to ensure efficient disinfection and dissolution of all organic content.

Certain *in vitro* studies suggest that high concentrations of NaOCl have a stronger effect on *E. faecalis* and *C. albicans* (28, 29, 34). In contrast, *in vivo* studies showed that both low and high concentrations have the same efficacy in eliminating microorganisms from the root canal (35). NaOCl can inactivate bacterial endotoxin, but this effect is much less than its antibacterial effect (36, 37).

By reducing the concentration of the solution and the time of irrigation, the ability of NaOCl to penetrate into dentinal tubules and disinfect them decreases (38, 39). With the addition of surface active substances which reduce the surface tension, the depth of penetration of NaOCl into the tubules and the dissolution rate of the tissue are increased (4, 40).

Compared to its superior effect in terms of antimicrobial effects, NaOCl has a strong cytotoxic effect (41, 42).

As a result of collagen and glycosaminoglycan degradation, NaOCl may affect the hardness, flexural strength and elasticity of the root dentin (43-46).

To be effective on the organic and inorganic components of the smear layer during endodontic treatment, NaOCl irrigation followed by a final irrigation with EDTA is recommended. In case of contact between NaOCl and EDTA and their interaction, the loss of NaOCl's active component chlorine occurs, thereby reducing the antimicrobial efficacy of NaOCl (47), as well as the solubility of vital and/or necrotic tissue (48). This reducing effect may even be caused by low concentrations of EDTA (49, 50). The interaction between NaOCl and CHX is spotted, which leads to discoloration of dentin and creation of an orange-brown residue containing para-chloroaniline, which has a carcinogenic effect (51). The interaction between NaOCl and CHX and the formation of those residues depends on the concentration of irrigants (52). After root canal irrigation with NaOCl, the root canal should be rinsed with distilled water (49) to prevent or at least to reduce the interaction with other irrigants. Despite the positive properties of NaOCl, including its antimicrobial property, ability to dissolve organic tissues and lubricating effect, it also has some disadvantages, namely, its toxicity, its corrosive effect on endodontic instruments, particularly on those manufactured of nickel-titanium, and its lack of an effect on the inorganic component of the smear layer (13, 53, 54).

CHLORHEXIDINE (CHX)

CHX is used in endodontic therapy for irrigation and intracanal medication, in the form of a solution or a gel, in variable concentrations from 0.2 to 2%. It is the most commonly used irrigant after the use of NaOCl and EDTA (6). CHX is recommended as an irrigant because of its low toxicity, broad spectrum of antimicrobial effect, and gradual and prolonged effect on microorganisms (28, 55).

CHX has wide antimicrobial effects, including effectiveness on G + and G- bacteria and fungi, especially on *E. faecalis* and *C. albicans* (56). CHX is a positively charged hydrophobic and lipophilic molecule that attaches to negatively charged phosphate groups on the cell wall (57), leading to changes in the osmotic balance of the cell (58, 59). The antimicrobial activity of CHX depends on the pH (optimum pH of approximately 5.5-7) (60) and on the concentration of the solution (14). Low concentrations of CHX (0.2%) produce a bacteriostatic effect, while high concentrations (2%) are bactericidal, causing cell damage, coagulation of cytoplasm, and precipitation of proteins and nucleic acids (61). *In vivo* and *in vitro* studies indicate that CHX has an anti-microbial effect similar to that of NaOCl, and it produces a greater effect on *E. faecalis* and some fusiform bacterial strains present in the infected root canal (15, 28, 55, 62-64).

CHX has the property of substantivity. Due to the cation structure of its molecules it is attached to negatively charged surfaces in the oral cavity, and is continuously released and produces a prolonged antimicrobial activity. The use of CHX may achieve long-term antimicrobial activity for up to 12 weeks (65). Substantivity depends on the presence of the CHX molecule that interacts with dentin (66).

CHX has minimal or no effects on the reduction of lipopolysaccharides (LPS, endotoxin, a component of the outer membrane of G- bacteria), which play a significant role in the pathogenesis of apical periodontitis, causing pain that occurs in cases of infection in the root canal (67, 68).

Unlike NaOCl, CXH is not capable of dissolving organic tissue, it is relatively safe when used as an irrigant, and it does not cause allergic reactions (69, 70).

Due to its broad antimicrobial spectrum, as well as an inability to dissolve organic tissue, it is proposed that CHX should be used as a final irrigant after irrigating with NaOCl and EDTA (71). The combination of NaOCl and CHX improves antimicrobial efficacy, and the use of CHX as a final irrigant extends its antimicrobial activity, due to its substantivity (72).

If contact occurs accidentally between CHX and NaOCl during irrigation, the formation of an orange-brown residue and the formation of a chemical smear layer occurs, which may exhibit cytotoxic potential (49, 51), block the dentinal tubules, impair adhesion of material for the definite obturation (73, 74), and cause a colour change of dentin (75-77). When CHX comes into contact with EDTA, the formation of a milky white precipitate occurs as a result of an acid-base reaction (49). To avoid or at least to reduce this formation, it is necessary to prevent mutual contact between the two irrigants by thorough rinsing of the root canal using distilled water (49).

ETHYLENEDIAMINETETRAACETIC ACID (EDTA)

In addition to NaOCl, one of the most commonly used irrigants is EDTA (6). It is used for the dissolution of the inorganic part of the smear layer. Its mineralytic effect is expressed through its ability to bind divalent and trivalent metal ions, such as Ca^{2+} and Fe^{3+}. One molecule of EDTA binds a maximum of four calcium ions, which provides a relatively stable, water-soluble chelated complex. It is usually used in concentrations of 15-17%, at a pH of 7-8 (6, 71). A final root canal irrigation with 5 ml 17% EDTA for 3

minutes effectively removes the smear layer (7). Although a concentration of 17% is sufficient and commonly used to remove the smear layer, some studies have shown that lower concentrations of EDTA (15%, 10%, 5%, 1%), after initial irrigation with NaOCl, also effectively remove the smear layer (78).

In addition to the effect on the smear layer, EDTA can cause demineralization of dentin. With increasing concentrations, pH levels and the time of the exposure of dentin to EDTA, the degree of dentin demineralization increases. Application of 10 ml 17% EDTA for one min effectively removes the smear layer. If the exposure time is extended to 10 min, a severe erosion of the peritubular and intratubular dentin may occur (79). A study that examined the effect of EDTA and the combination of EDTA and NaOCl on dentin in elderly and young patients showed that it is necessary to avoid prolonged exposure of old dentin to the combination of those irrigants, to reduce the risk of excessive erosion and demineralization. Both irrigants led to an increased brittleness of already sclerotic root dentin, and consequently increased the incidence of cracks during the functional loading of the root (80).

Chelating agents significantly reduce the micro hardness and pressure resistance of dentin, and this effect is most pronounced when EDTA is used as an irrigant, either alone or in combination with 2.5% NaOCl (80).

Chelating agents also reduce the resistance of the root to fracture, and the use of 17% EDTA for 10 min and 1% NaOCl for one minute reduces the resistance to root fracture by approximately 1.5 times, while lower concentrations of EDTA (5%) and shorter exposure times (one minute) cause a smaller reduction in resistance (81).

As a result of the removal of the smear layer and demineralization of dentin, EDTA causes changes in the permeability of dentin by formation of certain precipitates and by partial or complete obturation of the root dentin tubules (82, 83). Therefore, complete removal of residual EDTA solution is necessary, using an application of either deionized/distilled water or saline solution.

CITRIC ACID

Citric acid is a weak organic acid and is used to remove the inorganic part of the smear layer after initial rinsing of the canal (8). It is used at a concentration of 1-50%, but the most commonly used concentration is 10%.

The effect of H+ ions from the citric acid leads to the release of ions from the surface of hydroxyapatite dentin crystals, forming soluble chelate complexes (84, 85). The effectiveness of citric acid may be improved by increasing the concentration (86), reducing the pH (87) and extending the time of use (88).

In comparison with EDTA, citric acid removes the smear layer in an inappropriate way (89) and has a more pronounced erosive effect on root dentin (90). In a study by De-Deus and his associates in which they exposed dentin to 1% citric acid, 17% EDTA and 17% EDTAC, citric acid showed the strongest decalcification effect on root dentin (91).

If 19% citric acid is used, it significantly reduces the micro hardness of dentin compared to 17% EDTA (92). Further, citric acid, like EDTA, has poor antimicrobial activity (16, 93, 94).

In vitro studies indicate some cytotoxicity of citric acid. However, 10% citric acid shows a significant biocompatibility compared to 17% EDTA and 17% EDTA-T (11). Using NaOCl and citric acid does not cause the formation of precipitates (49).

MTAD

MTAD is the first irrigant on the market that can simultaneously perform disinfection of the canal system and remove the smear layer (17). It is a mixture of antibiotics (doxycycline, 3%), chelating agents (4.25% citric acid) and a detergent (Tween 80). It is sold under the name of the manufacturer, BioPure MTAD (Dentsply Tulsa Dental, Tulsa, OK, USA) and is prepared by mixing the liquid contained in the syringe with the powder contained in the bottle, immediately prior to its application. As a mixture, it has a shelf life of 48 h, which is considerably shorter than those of other irrigants used in endodontic practice (95). It does not possess the ability to dissolve organic tissue. It is recommended for use as the final irrigant after complete chemo-mechanical treatment of the root canal (17, 96--99). The irrigation protocol recommends the use of MTAD for 5 min, after an initial root canal irrigation with 1.3% NaOCl for 20 minutes (100).

In the available literature, there are no data on the exact mechanism of the effects of MTAD. The ability to remove the smear layer is attributed to the effect of citric acid and doxycycline, while its antibacterial effect is due to the effect of doxycycline, which is a tetracycline with a broad antibacterial spectrum, and exerts its bacteriostatic effect by inhibiting protein synthesis (101).

Despite the fact that MTAD has proven effectiveness against *E. faecalis* (17, 96, 97, 102), as well as effectiveness in the removal of the smear layer (103-105), in subsequent studies its antimicrobial efficiency has been challenged (106-108). When MTAD is applied after an initial irrigation with 1.3% NaOCl, its antimicrobial effect is reduced, probably due to oxidation of MTAD under the influence of NaOCl (109). The antibacterial efficiency of MTAD may be reduced due to the presence of dentin and serum albumins from the root canal (110). Its efficiency in eliminating *E. faecalis* biofilm is relatively low (100, 111, 112). *E. faecalis* biofilm is more difficult to remove and is more resistant to the effects of antimicrobials than planktonic bacteria (113). MTAD removes the smear layer more efficiently than EDTA, particularly in the apical third of the canal (114), where it causes less pronounced erosive changes to the dentin (104).

The low surface tension of MTAD (34.5 mJ / m2) (115) may provide a more complete contact of the irrigant with the dentin of the root canal, extending its ability to penetrate deeper into dentinal tubules, ensuring a more efficient removal of the smear layer, disinfecting canal walls and leading to better and easier diffusion of the components of intracanal medicaments.

MTAD has a lower cytotoxicity than most commonly used irrigants and intracanal medicaments in endodontic therapy, such as eugenol, hydrogen peroxide (3% solution), $Ca(OH)_2$ paste, NaOCl (5.25%) and EDTA, while its cytotoxic effect is greater than that of lower concentrations of NaOCl (2.63%, 1.31%, 0.66%) (116).

There is only one *in vivo* study showing that MTAD causes postoperative pain during endodontic therapy (117). Further studies are needed to confirm the efficacy of MTAD solution in *in vivo* conditions as well.

QMix

QMix is a new irrigant recently introduced to the market. In addition to a detergent, CHX and EDTA are also included in its composition (114). It combines the advantages of EDTA, contains a surfactant plus CHX and has a slight effect on dentin. Based on the manufacturer's recommendation, it should be used as a final irrigant for a period of 60-90s, following a 6.15% NaOCl irrigation. If NaOCl is used during the chemo-mechanical preparation, it is necessary to rinse the canal with saline or distilled water before using QMix.

A large number of studies indicate that QMix has the same efficiency for removal of the smear layer as EDTA (18, 9), and it exhibits a lower erosive effect on dentin (118). Studies by Dai et al. (114) and Eliot et al. (9) indicated that QMix removes the smear layer more effectively than EDTA.

QMix effectively eliminates *E. faecalis,* eroding its biofilm more quickly than 1% NaOCl and 2% CHX and to the same extent as 2% NaOCl (18). The bactericidal effect of QMix on a one-day old bacterial biofilm is the same as the effect of 6% NaOCl and is more efficient than a lower concentration of NaOCl or 2% CHX (119, 120).

In studies in which the ability of QMix to penetrate dentinal tubules was tested, it was observed that QMix may present its antimicrobial activity at the same depth as 6% NaOCl, within three minutes (119-121). Exposure of dentin tubules to QMix for one minute is more effective than using 2% CHX for three minutes or using lower concentrations of NaOCl (120). When the smear layer is present, the bactericidal effect of QMix inside the dentinal tubules is greater after exposure to 6% NaOCl for ten minutes than after the combination of 6% NaOCl, 17% EDTA and 2% CHX (121).

In addition to efficient removal of the smear layer, QMix increases the humidity of dentin more than EDTA, which has a poorer wetting power (122); this is probably due to the effect of the detergent in the QMix.

Table 1. Characteristics of endodontic irrigants

NaOCl	• effective antimicrobial agent • current irrigant of choice • organic tissue solvent • lubricates • toxic • corrosive effect • not substantive • removes only the organic part of the smear layer
CHX	• wide range of antimicrobial effects against G + and G- bacteria and fungi • substantivity in dentin for up to 12 weeks • dentin components, inflammatory exudate may inhibit the antibacterial activity • no ability to dissolve organic or inorganic tissue • biocompatibility
EDTA	• effectively removes the smear layer after the initial NaOCl irrigation • demineralization of dentin
Citric acid	• less removal of the smear layer compared with EDTA • stronger erosive effect on the root canal dentin compared to EDTA
MTAD	• antimicrobial properties • effective solution for removal of the smear layer when used along with NaOCl • less adverse effects on dentinal structure • good biocompatibility • no dissolution of organic tissue • high cost • reduced shelf life
QMix	• antibacterial efficacy • effective solution for removal of the smear layer when used along with NaOCl • ready for use, fast working • less demineralization of dentin compared to EDTA

QMix has a lower cytotoxicity than 17% EDTA, 2% CHX and 3% NaOCl (123). Unlike NaOCl, QMix leads to cell death more slowly, without any cell lysis (124). Currently, there are no clinical studies investigating the efficacy of QMix as a final irrigant.

During the application of QMix there is no development of a white precipitate, which typically occurs using a mixture of EDTA and CHX, nor is there formation of an orange-brown residue resulting from combining NaOCl and CHX (18). This is due to its uniform chemical composition.

CONCLUSION

In everyday clinical practice, the most frequently used irrigant is sodium hypochlorite, which despite its organolitic and antibacterial properties, does not completely remove the smear layer. To completely remove the smear layer, it is necessary to combine NaOCl with EDTA or another chelating agent, which act on the inorganic mineral component of the smear layer. In everyday clinical practice, a final irrigation with CHX is recommended because of its property of substantivity and of providing a prolonged an-

timicrobial effect after completion of the biomechanical preparation of the root canal.

By eliminating some of the drawbacks of currently used irrigants, a new generation of irrigants, such as MTAD and QMix, have appeared, whose application is the subject of our interest. These irrigants, in addition to having an impact on the smear layer, possess the ability to disinfect root canals. Although incapable of dissolving organic tissue, their use as the final canal irrigant is recommended, but only after prior irrigation with NaOCl has been completed.

Future research should focus on finding an irrigant that has the ability to dissolve tissue, remove the smear layer and exert an antibacterial effect.

ACKNOWLEDGMENTS AND FUNDING

We are grateful to the Faculty of Medical Sciences, University of Kragujevac, Serbia, for their financial and logistical support and for providing necessary guidance concerning project implementation. This work was supported by the Internal Project of the Faculty of Medical Sciences, University of Kragujevac, Serbia (JP 06/2016).

REFERENCES

1. Peters OA, Schönenberger K, Laib A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. Int Endod J 2001; 34:221-30.
2. Violich DR, Chandler NP. The smear layer in endodontics - a review. Int Endod J 2010; 43:2-15.
3. Shen Y, Gao Y, Lin J, Ma J, Wang Z, Haapasalo M. Methods and models to study irrigation. Endod Topics 2012; 27:3-34.
4. Haapasalo M, Shen Y, Qian W, Gao Y. Irrigation in endodontics. Dent Clin North Am 2010; 54:291-312.
5. Basrani B, Haapasalo M. Update on endodontic irigating solutions. Endod Topics 2012; 27:74-102.
6. Dutner J, Mines P, Anderson A. Irrigation trends among American Association of Endodontists members: a web-based survey. J Endod 2012; 38:37-40.
7. Mello I, Kammerer BA, Yoshimoto D, Macedo MC, Antoniazzi JH. Influence of final rinse technique on ability of ethylenediaminetetraacetic acid of removing smear layer. J Endod 2010; 36:512-4.
8. Herrera DR, Santos ZT, Tay LY, Silva EJ, Loguercio AD, Gomes BP. Efficacy of different final irrigant activation protocols on smear layer removal by EDTA and citric acid. Microsc Res Tech 2013; 76:364-9.
9. Eliot C, Hatton JF, Stewart GP, Hildebolt CF, Jane Gillespie M, Gutmann JL. The effect of the irrigant QMix on removal of canal wall smear layer: an ex vivo study. Odontology 2014; 102:232-40.
10. Kanjevac T, Tanasković I. The application of transmission electron microscopy as a method of characterization of cell populations. REDEOR, Venice, Italy, March 24-27, 2015. Abstract book, S3
11. Malheiros CF, Marques MM, Gavini G. In vitro evaluation of the cytotoxic effects of acid solutions used as canal irrigants. J Endod 2005; 31:746-8.
12. Kanjevac T, Milovanovic M, Milosevic-Djordjevic O, Tesic Z, Ivanovic M, Lukic A. Cytotoxicity of glass ionomer cement on human exfoliated deciduous teeth stem cells correlates with released Fluoride, Strontium and Aluminium ion concetrations. Arch Biol Sci 2015; 67:619-30.
13. Marais JT, Williams WP. Antimicrobial effectiveness of electro chemically activated water as an endodontic irrigation solution. Int Endod J 2001; 34:237-43.
14. Basrani B, Tjaderhane L, Santos JM, Pascon E, Grad H, Lawrence HP, et al. Efficacy of chlorhexidine and calcium hydroxide containing medicaments against Enterococcus faecalis in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003; 96: 618–24.
15. Vianna ME, Gomes BP, Berber VB, Zaia AA, Ferraz CC, de Souza Filho FJ. In vitro evaluation of the antimicrobial activity of chlorhexidine and sodium hypochlorite. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 97:79-84.
16. Arias-Moliz MT, Ferrer-Luque CM, Espigares-García M, Baca P. Enterococcus faecalis biofilms eradication by root canal irrigants. J Endod 2009; 35:711-4.
17. Torabinejad M, Shabahang S, Aprecio RM, Ketterin JD. The antimicrobial effect of MTAD: an in vitro investigation. J Endod 2003; 29:400–3.
18. Stojicic S, Shen Y, Qian W, Johnson B, Haapasalo M. Antibacterial and smear layer removal ability of a novel irrigant, QMiX. Int Endod J 2012; 45:363–71.
19. McDonnell G, Russell AD. Antiseptics and disinfectants: activity, action, and resistance. Clin Microbiol Rev 1999; 12:147-79.
20. Slaus G, Bottenberg P. A survey of endodontic practice amongst Flemish dentists. Int Endod J 2002; 35:759-67.
21. Clarkson RM, Podlich HM, Savage NW, Moule AJ. A survey of sodium hypochlorite use by general dental practitioners and endodontists in Australia. Aust Dent J 2003; 48:20-6.
22. Behrents KT, Speer ML, Noujeim M. Sodium hypochlorite accident with evaluation by cone beam computed tomography. Int Endod J 2012; 45:492-8.
23. Kleier DJ, Averbach RE, Mehdipour O. The sodium hypochlorite accident: experience of diplomates of the American Board of Endodontics. J Endod 2008; 34:1346-50.
24. Estrela C, Barbin E, Spanó J, Marchesan M, Pécora J. Mechanism of action of sodium hypochlorite. Braz Dent J 2002; 13:113–7.
25. Hand RE, Smith ML, Harrison JW. Analysis of the effect of dilution on the necrotic tissue dissolution property of sodium hypochlorite. J Endod 1978; 4:60–4.

26. Spangberg L, Engström B, Langeland K. Biologic effects of dental materials. 3.Toxicity and antimicrobial effect of endodontic antiseptics in vitro. Oral Surg Oral Med Oral Pathol 1973; 36:856-71.
27. Stojicic S, Zivkovic S, Qian W, Zhang H, HaapasaloM. Tissue dissolution by sodium hypochlorite: effect of concentration, temperature, agitation and surfactant. J Endod 2010; 36: 1558–62.
28. Gomes BP, Ferraz CC, Vianna ME, Berber VB, Teixeira FB, Souza-Filho FJ. In vitro antimicrobial activity of several concentrations of sodium hypochlorite and chlorhexidine gluconate in the elimination of Enterococcus faecalis. Int Endod J 2001; 34:424-8.
29. Radcliffe CE, Potouridou L, Qureshi R, Habahbeh N, Qualtrough A, Worthington H, et al. Antimicrobial activity of varying concentrations of sodium hypochlorite on the endodontic microorganisms Actinomyces israelii, A. naeslundii, Candida albicans and Enterococcus faecalis. Int Endod J 2004; 37:438-46.
30. Siqueira JF, Rôças IN, Favieri A, Lima KC. Chemomechanical reduction of the bacterial population in the root canal after instrumentation and irrigation with 1%, 2.5%, and 5.25% sodium hypochlorite. J Endod 2000; 26:331-4.
31. Moorer WR, Wesselink PR. Factors promoting the tissue dissolving capability of sodium hypochlorite. Int Endod J 1982; 15:187-96.
32. Slutzky-Goldberg I, Hanut A, Matalon S, Baev V, Slutzky H. The effect of dentin on the pulp tissue dissolution capacity of sodium hypochlorite and calcium hydroxide. J Endod 2013; 39:980-3.
33. Haapasalo HK, Sirén EK, Waltimo TM, Ørstavik D, Haapasalo MP. Inactivation of local root canal medicaments by dentine: an in vitro study. Int Endod J 2000; 33:126-31.
34. Waltimo TM, Ørstavik D, Sirén EK, Haapasalo MP. In vitro susceptibility of Candida albicans to four disinfectants and their combinations. Int Endod J 1999; 32:421-9.
35. Byström A, Sundqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. Int Endod J 1985; 18:35-40.
36. Martinho FC, Gomes BP. Quantification of endotoxins and cultivable bacteria in root canal infection before and after chemomechanical preparation with 2.5% sodium hypochlorite. J Endod 2008; 34:268-72.
37. Vianna ME, Horz HP, Conrads G, Zaia AA, Souza-Filho FJ, Gomes BP. Effect of root canal procedures on endotoxins and endodontic pathogens. Oral Microbiol Immunol 2007; 22:411-8.
38. Kuga MC, Gouveia-Jorge É, Tanomaru-Filho M, Guerreiro-Tanomaru JM, Bonetti-Filho I, Faria G. Penetration into dentin of sodium hypochlorite associated with acid solutions. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011; 112:155-9.
39. Zou L, Shen Y, Li W, Haapasalo M. Penetration of sodium hypochlorite into dentin. J Endod 2010; 36:793-6.
40. Cameron JA. The effect of a fluorocarbon surfactant on the surface tension of the endodontic irrigant, sodium hypochlorite. A preliminary report. Aust Dent J 1986; 31:364-8.
41. Barnhart BD, Chuang A, Lucca JJ, Roberts S, Liewehr F, Joyce AP. An in vitro evaluation of the cytotoxicity of various endodontic irrigants on human gingival fibroblasts. J Endod 2005; 31:613-5.
42. Alaçam T, Omürlü H, Ozkul A, Görgül G, Misirligil A. Cytotoxicity versus antibacterial activity of some antiseptics in vitro. J Nihon Univ Sch Dent 1993; 35:22-7.
43. Zaparolli D, Saquy PC, Cruz-Filho AM. Effect of sodium hypochlorite and EDTA irrigation, individually and in alternation, on dentin microhardness at the furcation area of mandibular molars. Braz Dent J 2012; 23:654-8.
44. Grigoratos D, Knowles J, Ng YL, Gulabivala K. Effect of exposing dentine to sodium hypochlorite and calcium hydroxide on its flexural strength and elastic modulus. Int Endod J 2001; 34:113-9.
45. Marending M, Paqué F, Fischer J, Zehnder M. Impact of irrigant sequence on mechanical properties of human root dentin. J Endod 2007; 33:1325-8.
46. Oyarzún A, Cordero AM, Whittle M. Immunohistochemical evaluation of the effects of sodium hypochlorite on dentin collagen and glycosaminoglycans. J Endod 2002; 28:152-6.
47. Grawehr M, Sener B, Waltimo T, Zehnder M. Interactions of ethylenediamine tetraacetic acid with sodium hypochlorite in aqueous solutions. Int Endod J 2003; 36:411-7.
48. Irala LE, Grazziotin-Soares R, Salles AA, Munari AZ, Pereira JS. Dissolution of bovine pulp tissue in solutions consisting of varying NaOCl concentrations and combined with EDTA. Braz Oral Res 2010; 24:271-6.
49. Prado M, Santos Júnior HM, Rezende CM, Pinto AC, Faria RB, Simão RA, et al. Interactions between irrigants commonly used in endodontic practice: a chemical analysis. J Endod 2013; 39:505-10.
50. Clarkson RM, Podlich HM, Moule AJ. Influence of ethylenediaminetetraacetic acid on the active chlorine content of sodium hypochlorite solutions when mixed in various proportions. J Endod 2011; 37:538-43.
51. Cintra LT, Watanabe S, Samuel RO, da Silva Facundo AC, de Azevedo Queiroz IO, Dezan-Júnior E, et al. The use of NaOCl in combination with CHX produces cytotoxic product. Clin Oral Investig 2014; 18:935-40.
52. Gasic J, Popovic J, Zivković S, Petrovic A, Barac R, Nikolic M. Ultrastructural analysis of the root canal walls after simultaneous irrigation of different sodium hypochlorite concentration and 0.2% chlorhexidine gluconate. Microsc Res Tech 2012;75:1099-103.
53. McComb D, Smith DC. A preliminary scanning electron microscopic study of root canals after endodontic procedures. J Endod 1975; 1:238-42.

54. Gernhardt CR, Eppendorf K, Kozlowski A, Brandt M. Toxicity of concentrated sodium hypochlorite used as an endodontic irrigant. Int Endod J 2004; 37:272-80.
55. Jeansonne MJ, White RR. A comparison of 2.0% chlorhexidine gluconate and 5.25% sodium hypochlorite as antimicrobial endodontic irrigants. J Endod 1994; 20:276-8.
56. Gomes BP, Vianna ME, Zaia AA, Almeida JF, Souza-Filho FJ, Ferraz CC. Chlorhexidine in endodontics. Braz Dent J 2013; 24:89-102.
57. Gomes BP, Souza SF, Ferraz CC, Teixeira FB, Zaia AA, Valdrighi L, et al. Effectiveness of 2% chlorhexidine gel and calcium hydroxide against Enterococcus faecalis in bovine root dentine in vitro. Int Endod J 2003; 36:267-75.
58. Hugo WB, Longworth AR. The effect of chlorhexidine on the electrophoretic mobility, cytoplasmic constituents, dehydrogenase activity and cell walls of Escherichia coli and Staphylococcus aureus. J Pharm Pharmacol 1966; 18:569–78.
59. Hugo W, Longworth A. Some aspects of the mode of action of chlorhexidine. J Pharm Pharmaco 1964; 16:751–8.
60. Siqueira JF Jr, Paiva SS, Rôças IN. Reduction in the cultivable bacterial populations in infected root canals by a chlorhexidine-based antimicrobial protocol. J Endod 2007; 33:541-7.
61. Jones CG. Chlorhexidine: is it still the gold standard? Periodontol 2000 1997; 15:55-62.
62. Gomes BP, Vianna ME, Matsumoto CU, Rossi V de P, Zaia AA, Ferraz CC, et al. Disinfection of gutta-percha cones with chlorhexidine and sodium hypochlorite. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2005; 100:512-7.
63. Siqueira JF Jr, Rocas IN, Paiva SS, Guimaraes-Pinto T, Magalhaes KM, Lima KC. Bacteriologic investigation of the effects of sodium hypochlorite and chlorhexidine during the endodontic treatment of teeth with apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007; 104:122-30.
64. Ercan E, Ozekinci T, Atakul F, Gül K. Antibacterial activity of 2% chlorhexidine gluconate and 5.25% sodium hypochlorite in infected root canal: in vivo study. J Endod 2004; 30:84-7.
65. Rosenthal S, Spangberg L, Safavi K. Chlorhexidine substantivity in root canal dentin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 98:488-92.
66. Mohammadi Z, Abbott PV. The properties and applications of chlorhexidine in endodontics. Int Endod J 2009; 42:288-302.
67. Buck RA, Cai J, Eleazer PD, Staat RH, Hurst HE. Detoxification of endotoxin by endodontic irrigants and calcium hydroxide. J Endod 2001; 27:325-7.
68. Gomes BP, Martinho FC, Vianna ME. Comparison of 2.5% sodium hypochlorite and 2% chlorhexidine gel on oral bacterial lipopolysaccharide reduction from primarily infected root canals. J Endod 2009; 35:1350-3.
69. Okino LA, Siqueira EL, Santos M, Bombana AC, Figueiredo JA. Dissolution of pulp tissue by aqueous solution of chlorhexidine digluconate and chlorhexidine digluconate gel. Int Endod J 2004; 37:38-41.
70. Hülsmann M, Rödig T, Nordmeyer S. Complications during root canal irrigation. Endod Topics 2007; 16:27–63.
71. Zehnder M. Root canal irrigants. J Endod 2006; 32:389-98.
72. Vianna ME, Gomes BPFA. Efficacy of sodium hypochlorite combined with chlorhexidine against Enterococcus faecalis in vitro. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 107:585-9.
73. Vivacqua-Gomes N, Ferraz CC, Gomes BP, Zaia AA, Teixeira FB, Souza-Filho FJ. Influence of irrigants on the coronal microleakage of laterally condensed gutta-percha root fillings. Int Endod J 2002; 35:791-5.
74. Do Prado M, Simao RA, Gomes BP. Evaluation of different irrigation protocols concerning the formation of chemical smear layer. Microsc Res Tech 2013; 76:196-200.
75. Basrani B, Lemonie C. Chlorhexidine gluconate. Aust Endod J 2005; 31:48-52.
76. Akisue E, Tomita VS, Gavini G, Poli de Figueiredo JA. Effect of the combination of sodium hypochlorite and chlorhexidine on dentinal permeability and scanning electron microscopy precipitate observation. J Endod 2010; 36:847-50.
77. Bui TB, Baumgartner JC, Mitchell JC. Evaluation of the interaction between sodium hypochlorite and chlorhexidine gluconate and its effect on root dentin. J Endod 2008; 34: 181–5.
78. Sen BH, Ertürk O, Pişkin B. The effect of different concentrations of EDTA on instrumented root canal walls. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009; 108:622-7.
79. Calt S, Serper A. Time-dependent effects of EDTA on dentin structures. J Endod 2002; 28:17–9.
80. Ozdemir HO, Buzoglu HD, Çalt S, Çehreli ZC, Varol E, Temel A. Chemical and ultramorphological effects of ethylenediaminetetraacetic acid and sodium hypochlorite on young and old root canal dentin. J Endod 2012; 38:204–8.
81. Uzunoglu E, Aktemur S, Uyanik MO, Durmaz V, Nagas E. Effect of ethylenediaminetetraacetic acid on root fracture with respect to concentration at different time exposures. J Endod 2012; 38:1110–3.
82. Fogel H, Pashley D. Dentin permeability: effects of endodontic procedures on root slabs. J Endod 1990; 16:442–5.
83. Guignes P, Faure J, Maurette A. Relationship between endodontic preparations and human dentin permeability measured in situ. J Endod 1996; 22:60–7.
84. Papagianni M. Advances in citric acid fermentation by Aspergillus niger: Biochemical aspects, membrane transport and modeling. Biotechnol Adv 2007; 25:244-63.

85. Dorozhkin SV. Surface Reactions of Apatite Dissolution. J Colloid Interface Sci 1997; 191:489-97.
86. Reis C, De-Deus G, Leal F, Azevedo É, Coutinho-Filho T, Paciornik S. Strong effect on dentin after the use of high concentrations of citric acid: an assessment with co-site optical microscopy and ESEM. Dent Mater 2008; 24:1608-15.
87. Haznedaroglu F. Efficacy of various concentrations of citric acid at different pH values for smear layer removal. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003; 96:340-4.
88. Machado-Silveiro LF, González-López S, González-Rodríguez MP . Decalcification of root canal dentine by citric acid, EDTA and sodium citrate. Int Endod J 2004; 37:365-9.
89. Khademi A, Feizianfard M. The effect of EDTA and citric acid on smear layer removal of mesial canals of first mandibular molars, a scanning electron microscopic study. J Res Med Sci 2004; 9:80-8.
90. Qian W, Shen Y, Haapasalo M. Quantitative analysis of the effect of irrigant solution sequences on dentin erosion. J Endod 2011; 37:1437–41.
91. De-Deus G, Reis C, Fidel S, Fidel RA, Paciornik S. Longitudinal and quantitative evaluation of dentin demineralization when subjected to EDTA, EDTAC, and citric acid: a co-site digital optical microscopy study. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008; 105:391-7.
92. Eldeniz AU, Erdemir A, Belli S. Effect of EDTA and citric acid solutions on the microhardness and the roughness of human root canal dentin. J Endod 2005; 31:107-10.
93. Siqueira JF Jr, Batista MM, Fraga RC, de Uzeda M. Antibacterial effects of endodontic irrigants on black-pigmented gram-negative anaerobes and facultative bacteria. J Endod 1998; 24:414-6.
94. Georgopoulou M, Kontakiotis E, Nakou M. Evaluation of the antimicrobial effectiveness of citric acid and sodium hypochlorite on the anaerobic flora of the infected root canal. Int Endod J 1994; 27:139-43.
95. Clarkson RM, Moule AJ, Podlich HM. The shelf-life of sodium hypochlorite irrigating solutions. Aust Dent J 2001; 46:269-76.
96. Shabahang S, Torabinejad M. Effect of MTAD on Enterococcus faecalis-contaminated root canals of extracted human teeth. J Endod 2003; 29:576–9.
97. Shabahang S, Pouresmail M, Torabinejad M. In vitro antimicrobial efficacy of MTAD and sodium hypochlorite. J Endod 2003; 450–2.
98. Baumgartner JC, Johal S, Marshall JG. Comparison of the antimicrobial efficacy of 1.3% NaOCl/Biopure MTAD to 5.25% NaOCl/15% EDTA for root canal irrigation. J Endod 2007; 33:48–51.
99. Beltz RE, Torabinejad M, Pouresmail M. Quantitative analysis of the solubilizing action of MTAD, sodium hypochlorite, and EDTA on bovine pulp and dentin. J Endod 2003; 29: 334–7.
100. Dunavant TR, Regan JD, Glickman GN, Solomon ES, Honeyman AL. Comparative evaluation of endodontic irrigants against Enterococcus faecalis biofilms. J Endod 2006; 32:527-31.
101. Haznedaroglu F, Ersev H. Tetracycline HCl solution as a root canal irrigant. J Endod 2001; 27:738–40.
102. Newberry BM, Shabahang S, Johnson N, Aprecio RM, Torabinejad M. The antimicrobial effect of Biopure MTAD on eight strains of Enterococcus faecalis: an in vitro investigation. J Endod 2007; 33:1352-4.
103. Davis JM, Maki J, Bahcall JK. An in vitro comparison of the antimicrobial effects of various endodontic medicaments on Enterococcus faecalis. J Endod 2007; 33:567-9.
104. Torabinejad M, Khademi AA, Babagoli J, Cho Y, Johnson WB, Bozhilov K, et al. A new solution for the removal of the smear layer. J Endod 2003; 29:170-5.
105. Torabinejad M, Cho Y, Khademi AA, Bakland LK, Shabahang S. The effect of various concentrations of sodium hypochlorite on the ability of MTAD to remove the smear layer. J Endod 2003; 29:233–9.
106. Kho P, Baumgartner JC. A comparison of the antimicrobial efficacy of NaOCl/Biopure MTAD versus NaOCl/EDTA against Enterococcus faecalis. J Endod 2006; 32:652–5.
107. Johal S, Baumgartner JC, Marshall JG. Comparison of the antimicrobial efficacy of 1.3% NaOCl/BioPure MTAD to 5.25% NaOCl/15% EDTA for root canal irrigation. J Endod 2007; 33:48–51.
108. Tay FR, Hiraishi N, Schuster GS, Pashley DH, Loushine RJ, Ounsi HF, et al. Reduction in antimicrobial substantivity of MTAD after initial sodium hypochlorite irrigation. J Endod 2006; 32:970–5.
109. Tay FR, Pashley DH, Loushine RJ, Doyle MD, Gillespie WT, Weller RN, et al. Ultrastructure of smear layer-covered intraradicular dentin after irrigation with BioPure MTAD. J Endod 2006; 32: 218–21.
110. Haapasalo M, Qian W, Portenier I, Waltimo T. Effects of dentin on the antimicrobial properties of endodontic medicaments. J Endod 2007; 33:917-25.
111. Clegg MS, Vertucci FJ, Walker C, Belanger M, Britto LR. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. J Endod 2006; 32:434–7.
112. Giardino L, Ambu E, Savoldi E, Rimondini R, Cassanelli C, Debbia EA. Comparative evaluation of antimicrobial efficacy of sodium hypochlorite, MTAD, and Tetraclean against Enterococcus faecalis biofilm. J Endod 2007; 33:852–5.
113. Svensäter G, Bergenholtz G. Biofilms in endodontic infections. Endod Topics 2004; 9:27–36.
114. Dai L, Khechen K, Khan S, Gillen B, Loushine BA, Wimmer CE, et al. The effects of QMiX, an experimental antibacterial rootcanal irrigant, on removal of canal wall smear layerand debris. J Endod 2011; 37: 80–4.
115. Giardino L, Ambu E, Becce C, Rimondini L, Morra M. Surface tension comparison of four common root canal irrigants and two new irrigants containing antibiotic. J Endod 2006; 32:1091-3.

116. Zhang W, Torabinejad M, Li Y. Evaluation of cytotoxicity of MTAD using the MTT-tetrazolium method. J Endod 2003; 29:654-7.
117. Torabinejad M, Shabahang S, Bahjri K. Effect of MTAD on postoperative discomfort: a randomized clinical trial. J Endod 2005; 31:171-6.
118. Aranda-Garcia AJ, Kuga MC, Chavéz-Andrade GM, Kalatzis-Sousa NG, Hungaro Duarte MA, Faria G, et al. Effect of final irrigation protocols on microhardness and erosion of root canal dentin. Microsc Res Tech 2013; 76:1079-83.
119. Wang Z, Shen Y, Haapasalo M. Effectiveness of endodontic disinfecting solutions against young and old Enterococcus faecalis biofilms in dentin canals. J Endod 2012; 38:1376-9.
120. Ma J, Wang Z, Shen Y, Haapasalo M. A new noninvasive model to study the effectiveness of dentin disinfection by using confocal laser scanning microscopy. J Endod 2011; 37:1380-5.
121. Wang Z, Shen Y, Haapasalo M. Effect of smear layer against disinfection protocols on Enterococcus faecalis-infected dentin. J Endod 2013; 39:1395-400.
122. Ballal NV, Tweeny A, Khechen K, Prabhu KN, Satyanarayan, Tay FR. Wettability of root canal sealers on intraradicular dentine treated with different irrigating solutions. J Dent 2013; 41:556-60.
123. Chandrasekhar V, Amulya V, Rani VS, Prakash TJ, Ranjani AS, Gayathri C. Evaluation of biocompatibility of a new root canal irrigant Q Mix™ 2 in 1- An in vivo study. J Conserv Dent 2013; 16:36-40.
124. Alkahtani A, Alkahtany SM, Mahmood A, Elsafadi MA, Aldahmash AM, Anil S. Cytotoxicity of QMix™ endodontic irrigating solution on human bone marrow mesenchymal stem cells. BMC Oral Health 2014; 14:27.

SYNTHESIS AND CHARACTERIZATION OF ZINC(II)-COMPLEXES WITH S-ALKYL DERIVATIVES OF THIOSALICYLIC ACID

Milos V. Nikolic[1], Marina Z. Mijajlovic[1], Dusan Lj. Tomovic[1], Andriana M. Bukonjic[1],
Verica V. Jevtic[2], Zoran R. Ratkovic[2], Srecko R. Trifunovic[2], Gordana P. Radic[1]
[1]University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Pharmacy
[2]University of Kragujevac, Serbia, Faculty of Science, Department of Chemistry

SINTEZA I KARAKTERIZACIJA CINK(II)-KOMPLEKSA SA NEKIM S-ALKIL DERIVATIMA TIOSALICILNE KISELINE

Miloš V. Nikolić[1], Marina Ž. Mijajlović[1], Dušan Lj. Tomović[1], Andriana M. Bukonjić[1],
Verica V. Jevtić[2], Zoran R. Ratković[2], Srećko R. Trifunović[2], Gordana P. Radić[1]
[1]Univerzitet u Kragujevcu, Srbija, Fakultet medicinskih nauka, Odsek za farmaciju
[2] Univerzitet u Kragujevcu, Srbija, Prirodno-matematički fakultet, Institut za hemiju

Received / Primljen: 02.02.2017. Accepted / Prihvaćen: 12.02.2017.

ABSTRACT

*New zinc(II)-complexes with S-alkyl derivatives of thiosalicylic acid (alkyl = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) have been synthesized and characterized by elemental microanalysis, IR spectroscopy, and ^{1}H and ^{13}C NMR spectroscopy. The S-alkyl derivatives of thiosalicylic acid were prepared by alkylation of thiosalicylic acid by adding alkyl halides to an alkaline water-ethanol solution, while the corresponding zinc(II)-complexes were obtained via the direct reaction of $ZnCl_2$ with S-alkyl derivatives of thiosalicylic acid in water. Based on the microanalysis results and the IR and NMR spectra of the S-alkyl derivatives of thiosalicylic acid and the corresponding zinc(II)-complexes, we concluded that the ligands are bidentately coordinated to the zinc(II)-ion.*

Keywords: *S-alkyl derivatives of thiosalicylic acid, zinc(II)-complexes, elemental microanalysis, IR and NMR spectroscopy*

SAŽETAK

*Novi cink(II)-kompleksi sa S-alkil derivatima tiosalicilne kiseline (alkil = benzil-(**L1**), metil-(**L2**), etil-(**L3**), propil-(**L4**), butil-(**L5**)) su sintetisani i okarakterisani na osnovu rezultata elementalne mikroanalize, IR, ^{1}H i ^{13}C NMR spektroskopije. S-alkil derivati tiosalicilne kiseline dobijeni su reakcijom alkilovanja tiosalicilne kiseline odgovarajućim alkil-halogenidima u baznom rastvoru voda-etanol dok su odgovarajući cink(II)-kompleksi dobijeni direktnom reakcijom $ZnCl_2$ i S-alkil derivata tiosalicilne kiseline u vodenom rastvoru. Na osnovu rezultata mikroanalize i infracrvenih i nuklearno-magnetno rezonancionih spektara S-alkil derivata tiosalicilne kiseline i odgovarajućih Zn(II)-kompleksa zaključili smo da su se molekuli liganada koordinovali bidentatno za cink(II)-jon.*

Ključne reči: *S-alkil derivati tiosalicilne kiseline, cink(II)-kompleksi, elementalna mikroanaliza, IR i NMR spektroskopija*

ABBREVIATIONS

CuZnSOD – copper-zinc superoxide dismutase
DNA - deoxyribonucleic acid
DMSO-d_6 - deuterated dimethyl sulfoxide
IR - infrared
LiOH - lithium hydroxide
NMR - nuclear magnetic resonance
TMS - tetramethylsilane
Zn – zinc
$ZnCl_2$ – zinc chloride

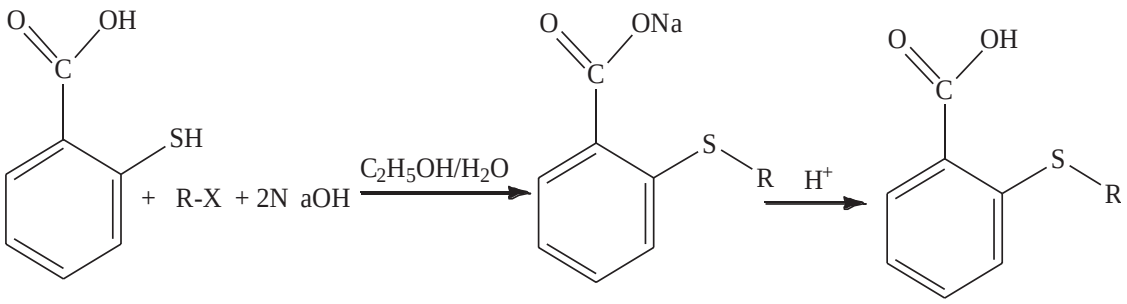
INTRODUCTION

Zinc is the most abundant trace intracellular element that plays a role in a wide range of cellular processes, including cell proliferation, reproduction, immune function, and defence against free radicals (1-4). Furthermore, the bioessential metal zinc is required for the catalytic activity of more than 300 specific enzymes and over 2000 zinc-associated transcription factors including DNA binding proteins with zinc fingers. Some well-studied zinc metalloenzymes include alcohol dehydrogenase, carbonic anhydrase, alkaline phosphatase and ribonucleic acid polymerases. Zinc also plays a structural role by stabilizing the tertiary structure of zinc metalloenzymes and other critical proteins. For example, zinc is required to stabilize the enzyme CuZnSOD (5,6).

Unlike iron and copper, zinc does not participate in redox reactions but rather functions as a Lewis acid to accept

sciendo
UDK: 547.587.11; 546.47 / Ser J Exp Clin Res 2018; 19 (2): 113-117
DOI: 10.1515/SJECR-2017-0005

Corresponding author: Gordana P. Radic
University of Kragujevac, Serbia, Faculty of Medical Sciences, Department of Pharmacy
Tel./fax: + 381 34306800; E-mail address: vasic_gordana@yahoo.com



Scheme 1. The preparation of the S-alkyl derivatives of thiosalicylic acid.
R= benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)

a pair of electrons. The coordination of zinc by four amino acid side chains to form zinc finger motifs facilitates stable protein folding to form biologically active proteins. Zinc is also known to play a direct role in the regulation of gene expression, although this role is less studied than its catalytic and structural functions (7).

Thiosalicylic acid and its derivatives are used in chemical analysis (8-11), in dermatology (12,13), and in the treatment of various diseases (14,15).

The synthesis and characterization of zinc(II)-complexes with thiosalicylic acid preceding this study focused on interactions of bioessential metals with biologically and pharmacologically active ligands. The zinc salt of thiosalicylic acid, which was isolated by the reaction of zinc chloride, thiosalicylic acid and sodium hydroxide in an alcohol-water solution, (16) has been applied to the treatment of acne and seborrheic dermatitis (17). When zinc acetate reacts with a solution of thiosalicylic acid in sodium acetate, a white precipitate of $[Zn(SC_6H_4CO_2]$ is formed, which is insoluble in all common solvents except pyridine and DMSO, in which it is more soluble, yielding the composition product $[Zn(SC_6H_4CO_2py]$ (18). Coordination chemistry of zinc with thiosalicylate and ancillary donor ligands has also been investigated (19). In more recent work, dimeric Zn(II)-complexes with 1,10-phenanthroline was structurally characterized and found to contain carboxylate bridges (20).

The aim of our present study was to synthesize and characterize five new zinc(II)-complexes with S-alkyl derivatives of thiosalicylic acid (alkyl = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)). The chemical characterization of S-alkyl derivatives of thiosalicylic acid has been previously published (21,22). The composition and structure of synthesized complexes was predicted based on elemental microanalysis and infrared and nuclear magnetic resonance spectra.

MATERIALS AND METHODS

Materials and measurements

All chemicals were obtained commercially and used without further purification. Elemental microanalyses were performed on a Vario III CHNOS Elemental Analyzer, Elemental Analysensysteme GmbH. For the infrared spectra, a Perkin-Elmer Spectrum One FT-IR spectrometer was employed. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini-200 NMR spectrometer using TMS in DMSO-d_6 as an internal reference at 22°C and with 10 mM solutions of the complexes.

Syntheses

General procedure for the synthesis of S-alkyl derivatives of thiosalicylic acid (L1)-(L5)

The S-alkyl derivatives of thiosalicylic acid ligands (alkyl = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) were prepared (22) by alkylation of thiosalicylic acid via addition of the corresponding alkyl halide in an alkaline water-ethanol solution (Scheme 1).

Preparation of $[Zn(S\text{-}bz\text{-}thiosal)_2]$ (C1), a zinc(II)-complex with the S-benzyl derivative of thiosalicylic acid

$ZnCl_2$ (0.1000 g, 0.7337 mmol) was dissolved in 10 cm^3 of water in a steam bath, and the S-benzyl derivative of thiosalicylic acid (0.3585 g, 1.4674 mmol) was added to the solution. The resulting mixture was stirred for 2 h, and during this time, an aqueous solution of LiOH (0.0352 g, 1.4674 mmol in 10 cm^3 of water) was introduced. The complex $[Zn(S\text{-}bz\text{-}thiosal)_2]$ (C1) as a white precipitate was filtered, washed with water and air-dried, with a yield of 0.25 g (61.22%). *Anal.* Calc. for $[Zn(S\text{-}bz\text{-}thiosal)_2]$ = $ZnC_{28}H_{22}O_4S_2$ (Mr = 551.978): C, 60.92; H, 4.02; S, 11.62. Found: C, 60.53; H, 3.94; S, 11.71. IR (KBr, cm^{-1}): 3428, 3059, 2922, 1598, 1580, 1437, 1403, 1282, 1259, 1063, 1044, 846, 744, 697. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 4.03 (s, 4H, CH_2), 7.26-8.25 (m, 18H, Ar и bz). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 26 (CH_2), 125; 126.4; 126.8, 127.2; 127.3; 128.9; 133.5; 134.3; 137.7, 138.6 (Ar и bz); 169.2 (COO^-).

Preparation of $[Zn(S\text{-}met\text{-}thiosal)_2]$ (C2), a zinc(II)-complex with the S-methyl derivative of thiosalicylic acid

The complex $[Zn(S\text{-}met\text{-}thiosal)_2]$ (**C2**) was prepared as described above using the S-methyl derivative of thiosalicylic acid (0.2467 g, 1.4674 mmol) instead of the

S-benzyl derivative of thiosalicylic acid, with a yield of 0.19 g (64.29%). *Anal.* Calc. for [Zn(S-met-thiosal)$_2$] = $ZnC_{16}H_{14}O_4S_2$ (Mr = 399.794): C, 48.06; H, 3.53; S, 16.04. Found: C, 47.69; H, 3.78; S, 15.71. IR (KBr, cm^{-1}): 3436, 2917, 2859, 1593, 1576, 1435, 1399, 1280, 1256, 1156, 1065, 953, 847, 744, 654. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 2.47 (s, 6H, CH_3), 7.42-8.30 (m, 8H, Ar). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 15.6 (CH_3), 126; 126.3; 126.8; 133.4; 134.3; 137.2 (Ar), 169.4 (COO^-).

Preparation of [Zn(S-et-thiosal)$_2$] (C3), a zinc(II)-complex with the S-ethyl derivative of thiosalicylic acid

The complex [Zn(S-et-thiosal)$_2$] (**C3**) was prepared as described above using the S-ethyl derivative of thiosalicylic acid (0.2673 g, 1.4674 mmol) instead of the S-benzyl derivative of thiosalicylic acid, with a yield of 0.18 g (58.47%). *Anal.* Calc. for [Zn(S-et-thiosal)$_2$] = $ZnC_{18}H_{18}O_4S_2$ (Mr = 427.846): C, 50.53; H, 4.24; S, 14.99. Found: C, 50.17; H, 4.07; S, 14.88. IR (KBr, cm^{-1}): 3432, 2954, 2765, 1595, 1563, 1436, 1404, 1273, 1149, 1122, 1054, 995, 871, 784, 739, 693, 655. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 1.27 (t, 6H, CH_3), 2.81 (q, 4H, CH_2), 7.43–8.27 (m, 8H, Ar). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 13.9 (CH_3), 13 (CH_2), 125.1; 126.6; 126.5; 133.3; 134.2; 137.1 (Ar), 169.3 (COO^-).

Preparation of [Zn(S-pr-thiosal)$_2$] (C4), a zinc(II)-complex with the S-propyl derivative of thiosalicylic acid

The complex [Zn(S-pr-thiosal)$_2$] (**C4**) was prepared as described above using the S-propyl derivative of thiosalicylic acid (0.2879 g, 1.4674 mmol) instead of the S-benzyl derivative of thiosalicylic acid, with a yield of 0.20 g (62.19%). *Anal.* Calc. for [Zn(S-pr-thiosal)$_2$] = $ZnC_{20}H_{22}O_4S_2$ (Mr = 455.898): C, 52.69; H, 4.86; S, 14.07. Found: C, 52.27; H, 4.78; S, 13.91. IR (KBr, cm^{-1}): 3443, 3061, 2965, 2931, 2863, 2559, 1591, 1562, 1462, 1435, 1312, 1294, 1252, 1137, 1092, 1055, 867, 794, 755, 692, 652. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 0.90 (t, 6H, CH_3), 1.35 (m, 4H, CH_2), 2.77 (t, 4H, CH_2), 7.41–8.32 (m, 8H, Ar). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 13.0 (CH_3), 24.2 (CH_2), 22 (CH_2), 125.3; 126.6; 126.5; 133.4; 134.2; 138.7 (Ar), 169.1 (COO^-).

Preparation of [Zn(S-bu-thiosal)$_2$] (C5), a zinc(II)-complex with the S-butyl derivative of thiosalicylic acid

The complex [Zn(S-bu-thiosal)$_2$] (**C5**) was prepared as described above using the S-butyl derivative of thiosalicylic acid (0.3085 g, 1.4674 mmol) instead of the S-benzyl derivative of thiosalicylic acid, with a yield of 0.21 g (60.12%). *Anal.* Calc. for [Zn(S-bu-thiosal)$_2$] = $ZnC_{22}H_{26}O_4S_2$ (Mr = 483.950): C, 54.60; H, 5.42; S, 13.25. Found: C, 54.33; H, 5.19; S, 13.17. IR (KBr, cm^{-1}): 3436, 3053, 2952, 2934, 2867, 2529, 1614, 1594, 1582, 1565, 1467, 1430, 1408, 1312, 1291, 1252, 1139, 1098, 1063, 1051, 918, 853, 754, 733, 697, 652, 551. ^{1}H NMR (200 MHz, DMSO-d_6, δ ppm): 0.88 (t, 6H, CH_3), 1.44 (m, 4H, CH_2), 1.60 (m, 4H, CH_2), 2.77 (t, 4H, CH_2), 7.42-8.28 (m, 8H, Ar). ^{13}C NMR (50 MHz, DMSO-d_6, δ ppm): 13.4 (CH_3), 21.6 (CH_2), 32 (CH_2), 20 (CH_2), 124.9; 126.6; 126.8; 133.3; 134.2; 138.5 (Ar), 168.8 (COO^-).

RESULTS AND DISCUSSION

The S-alkyl derivatives of thiosalicylic acid ligands (R = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) were prepared by alkylation of thiosalicylic acid via addition of the corresponding alkyl halide to an alkaline water-ethanol solution (Scheme 1).

The corresponding zinc(II)-complexes were obtained via the direct reaction of $ZnCl_2$ with the appropriate S-alkyl derivative of thiosalicylic acid (in a molar ratio 1:2) in water.

Infrared spectra of the obtained complexes were analyzed to determine the coordination mode of the ligands to the zinc (II) ion. Based on previous results (18-20), we expected a bidentate coordination of S-alkyl derivatives of thiosalicylic acid through S and O donor atoms. Asymmetric stretching frequencies of the carboxyl groups in the infrared spectrum of isolated ligands (22) are observed at lower values than expected (from 1700 to 1750 cm^{-1}) (23-25), which could be explained by the presence of large R-S groups in the *ortho* position. The positions of these frequencies in the infrared spectrum of the corresponding zinc(II)-complexes (C1-C5) are located in the expected region (1580 to 1620 cm^{-1}), which confirms their coordination to the zinc(II)-ion (Table 1).

Hydrogen and carbon chemical shifts of the S-alkyl derivatives of thiosalicylic acid and the corresponding zinc(II)-complexes were found at almost the same positions. Only minor differences in the chemical shifts of the carbon atoms from the carboxyl group of the S-alkyl derivative of thiosalicylic acid and the corresponding zinc(II)-complexes were observed. These differences in the chemical shifts of the carboxyl group may be explained by ligand coordination through the oxygen atom of the carboxyl group to the zinc (II) ion (Table 2).

Based on the microanalysis results and the IR and NMR spectra of the ligands and the corresponding Zn(II)-complexes, we concluded that the ligands are bidentately coordinated to the zinc(II)-ion through S and O donor atoms. However, based on these results, we could not conclude anything about the complex geometry. The precise molecular structure of the obtained zinc(II)-complexes can only be determined using X-ray analysis.

CONCLUSION

Zinc(II)-complexes with S-alkyl derivative of thiosalicylic acid (R = benzyl-(**L1**), methyl-(**L2**), ethyl-(**L3**), propyl-(**L4**), butyl-(**L5**)) have been synthesized and characterized by microanalysis, infrared spectroscopy, and ^{1}H and ^{13}C NMR

Table 1. The most important infrared bands (cm^{-1}) of the investigated compounds

Compound	-COO- (as)
[Zn(S-bz-thiosal)$_2$] (C1)	1598, 1580
[Zn(S-met-thiosal)$_2$] (C2)	1593
[Zn(S-et-thiosal)$_2$] (C3)	1595
[Zn(S-pr-thiosal)$_2$] (C4)	1591
[Zn(S-bu-thiosal)$_2$] (C5)	1614,1594

Table 2. ^{1}H and ^{13}C NMR spectra of the ligands (22) and corresponding Zn(II)-complexes

Ligands		^{1}H	^{13}C	Zn(II)-complexes		^{1}H	^{13}C
(L1)	CH_2-bz Ar and bz COOH	4.17 7.21-8.14 -	35.9 124.1-141.3 167.5	(C1)	CH_2-bz Ar и bz COOH	4.03 7.26-8.25 -	26.0 125.0-138.6 169.2
(L2)	CH_3- Ar COOH	2.48 7.16-8.18 -	15.6 123.5-144.4 171.6	(C2)	CH_3- Ar COOH	2.47 7.42-8.30 -	15.6 126.0-137.2 169.4
(L3)	CH_3- CH_2- Ar COOH	1.42 2.97 7.16-8.17 -	13.1 26.2 124.0-142.6 171.4	(C3)	CH_3- CH_2- Ar COOH	1.27 2.81 7.43-8.27 -	13.9 13.0 125.1-137.1 169.3
(L4)	CH_3- CH_2- CH_2- Ar COOH	1.1 1.74 2.92 7.15-8.15 -	13.8 21.6 34.1 123.8-143.1 171.6	(C4)	CH_3- CH_2- CH_2- Ar COOH	0.90 1.35 2.77 7.41-8.32 -	13.0 24.2 22.0 125.3-138.7 169.1
(L5)	CH_3- CH_2- CH_2- CH_2- Ar COOH	0.96 1.46 1.78 2.94 7.15-8.16 -	13.7 22.3 30.2 31.9 123.8-143.1 171.4	(C5)	CH_3- CH_2- CH_2- CH_2- Ar COOH	0.88 1.44 1.60 2.77 7.42-8.28 -	13.4 21.6 32.0 20.0 124.9-138.5 168.8

spectroscopy. Based on the microanalysis and spectroscopic results of the obtained compounds, we concluded that the ligands are bidentately coordinated to the zinc(II)-ion, but we could not yet conclude anything about the complex geometry.

Acknowledgements

This work was financially supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Projects 172016 and 172034).

REFERENCES

1. Broadley MR, White PJ, Hammond JP, Zelko I, Lux A. Zinc in plants. New Phytologist. 2007; 173(4): 677-702.
2. Bray TM, Bettger WJ. The physiological role of zinc as an antioxidant. Free Radical Biology and Medicine. 1990; 8(3): 281-91.
3. Powell SR. The antioxidant properties of zinc. The Journal of nutrition. 2000; 130(5): 1447-54.
4. Dreosti IE. Zinc and the gene. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 2001; 475(1): 161-7.
5. Prasad AS. Zinc deficiency in humans: a neglected problem. Journal of the American college of Nutrition. 1998; 17(6): 542-3.
6. Prasad AS, Kucuk O. Zinc in cancer prevention. Cancer and metastasis Reviews. 2002; 21(3-4): 291-5.
7. McCall KA, Huang CC, Fierke CA. Function and mechanism of zinc metalloenzymes. The Journal of nutrition. 2000; 130(5): 1437-46.
8. Chhakkar Ak, Kakkar LR. Extractive-spectrophotometric method for the determination of palladium using thiosalicylic acid and hexylamine. Fresenius Journal of analytical Chemistry. 1993; 347(12): 483-85.
9. Gregory GREC and Jeffery PG. Salicylideneamino-2-thiophenol—a new reagent for the photometric determination of tin: application to the analysis of ores, rocks and minerals. Analyst. 1967; 92: 293-299.
10. Gismera MJ, Procopio JR, Sevilla MT, Hernandez L. Copper(II) ion-selective electrodes based on dithiosalicylic and thiosalicylic acids. Electroanalysis. 2003; 15 (2): 126-132.
11. Aydin M, Arsu N, Yagci Y. One-component biomolecular photoinitiating systems. Macromol Rapid Commun. 2003; 24: 718–723.
12. Shander D, Ahluwalia G, Grosso D. Us Patent 5411991.
13. Tarbet B. Skin disorders, therapy using fungicides (2003). U.S. Patent Application No. 10/706,708.
14. Jacobelli H. US Patent 20050267095.
15. Halaschek-Wiener J, Kloog Y, Wacheck V, Jansen B. Farnesyl thiosalicylic acid chemosensitizes human melanoma in vivo. J Invest Dermatol. 2003; 120(1): 109-15.
16. Al-Niaimi NS, Al-Saadi BM. Thiosalicylic acid complexes of divalent zinc, cadmium, mercury and lead. Journal of Inorganic and Nuclear Chemistry. 1974; 36(7): 1617-22.
17. Al-Niaimi NS, Al-Saadi BM. Thiosalicylic acid complexes of divalent zinc, cadmium, mercury and lead. Journal of Inorganic and Nuclear Chemistry. 1974; 36(7): 1617-22.
18. Duran N, Clegg W, Cucurull-Sánchez L, Coxall RA, Jiménez HR, Moratal JM, Lloret F, González-Duarte P. Unprecedented stabilization of cobalt(II) in a tetrahedral S_2O_2 environment: the use of a redox-noninnocent ligand. Inorganic chemistry. 2000; 39(21): 4821-32.

19. Abdel-Mawgoud AM, Abdel-Hamid R. Cobalt(II), copper(II), zinc(II)-amino and thiosalicylic acids ternary complexes. Monatshefte für Chemie 1987; 118(11): 1219-23.
20. Dai YM, Huang JF, Shen HY. Bis(1, 10-phenanthroline-κ2N, N)bis(thiosalicylato-1κ2O, O': 2κ2O', S)dizinc(II). Acta Crystallographica Section E: Structure Reports Online. 2005; 61(12): 2491-2.
21. Smith DJ, Yap GP, Kelley JA, Schneider JP. Enhanced stereoselectivity of a Cu(II) complex chiral auxiliary in the synthesis of Fmoc-L-γ-carboxyglutamic acid. J Org Chem. 2011; 76(6):1513-20.
22. Radić GP, Glođović VV, Radojević ID, Stefanović OD, Čomić LjR, Ratković ZR, Valkonen A, Rissanen K, Trifunović SR. Synthesis, characterization and antimicrobial activity of palladium(II) complexes with some alkyl derivates of thiosalicylic acids. Crystal structure of bis(S-benzil-thiosalicylate)-palladium(II)complex, [Pd(S-bz-thiosal)$_2$]. Polyhedron. 2012; 31:69-76.
23. Schoenberg LN, Cooke DW, Liu CF. Nuclear magnetic resonance determination of the absolute configuration of complexes of cobalt(III) with asymmetric tetradentate ligands. Inorg Chem. 1968; 7: 2386-93.
24. Swaminathan K, Busch DH. The synthesis and infrared absorption spectra of complexes of cobalt with pentadentate propylenediaminetetraacetic acid. J Inorg Nucl Chem. 1961; 20(1): 159-63.
25. Nakamoto K. Infrared spectra of the inorganic and coordination compounds. New York: Willey; 1963.

EFFECTS OF CORIANDRUM SATIVUM EXTRACT AND SIMVASTATIN IN ISOPRETERENOL INDUCED HEART FAILURE IN RATS

Aisha Siddiqi[1], Adila Parveen[2], Neha Dhyani[1], M. Ejaz Hussain[2] and Mohammad Fahim[1]
[1]Department of Physiology, Hamdard Institute of Medical Sciences and Research, Jamia Hamdard (Hamdard University), Hamdard Nagar, New Delhi-110062, India
[2]Centre for Physiotherapy and Rehabilitation Sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi-110025, India

EFEKTI EKSTRAKTA KORIJANDERA I SIMVASTATINA NA IZOPROTERENOLOM-INDUKOVANU SRČANU INSUFICIJENCIJU KOD PACOVA

Aisha Siddiqi[1], Adila Parveen[2], Neha Dhyani[1], M. Ejaz Hussain[2] и Mohammad Fahim[1]
[1]Katedra za fiziologiju, Hamdard institut medicinskih nauka i istraživanja, Jamia Hamdard (Univerzitet Hamdard), Hamdard Nagar, Nju Delhi-110062, Indija
[2]Centar za fizioterapiju i rehabilitaciju, Jamia Millia Islamia, Jamia Nagar, Nju Delhi-110025, Indija

Received / Primljen: 11.10.2017 Accepted / Prihvaćen: 06.12.2017

ABSTRACT

Heart failure is a syndrome, caused due to structural and functional cardiac abnormalities, characterized by changes in the hemodynamic and neurohumoral mechanisms. It is becoming a major health burden worldwide. More effective therapies are desperately needed. Coriandrum sativum (C. sativum), a traditional spice crop has been known to possess many biological and medicinal properties. The present study was designed to investigate the cardioprotective efficacy of C. sativum in rat model of isoproterenol induced heart failure. Heart failure was produced by injecting isoproterenol subcutaneously (85 mg/kg twice at an interval of 24 h). Oral efficacy of seed extract was assessed on hemodynamic profile, antioxidant enzyme activities, lipid peroxidation, lipid profile, atherogenic indices, mRNA and protein expression of endothelin receptors (ET_A and ET_B) and histopathology. Treatment of heart failure rats with C. sativum orally (1g/kg b.wt) improved the altered hemodynamics, restored the cardiac antioxidant enzymes armory, attenuated oxidative stress, improved lipid profile, lowered atherogenic indices, decreased the levels of ET_A and ET_B receptor mRNA and protein, and restored the cardiac morphology. In conclusion, our results suggest C. sativum to be a cardioprotective agent in heart failure, possibly by the virtue of its ability to alleviate oxidative stress, improve lipid profile and endothelial dysfunction.

Keywords: *Coriandrum sativum, oxidative stress, heart failure, endothelial dysfunction, endothelin receptors*

SAŽETAK

Insuficijencija srca je sindrom uzrokovan strukturnim i funkcionalnim abnormalnostima srca, a karakteriše se promenama hemodinamskih i neurohumoralnih mehanizama. Insuficijencija srca predstavlja veliki zdravstveni problem širom sveta i zbog toga su neophodne efikasnije terapije. Korijander (C. sativum) je tradicionalno začinsko bilje za koje se zna da poseduje mnoga biološka i terapijska svojstva. Cilj ove studije bio je da ispita kardioprotektivne efekte C. sativuma na modelu srčane insuficijencije izazvane izoproterenom kod pacova. Srčana insuficijencija izazvana je subkutanom injekcijom izoproterenola (85 mg/kg dva puta u intervalu od 24 h). Efikasnost per os primenjenog ekstrakta semena korijandera ispitivana je na hemodinamski profil, aktivnost antioksidacionih enzima, peroksidaciju lipida, lipidni profil, aterogeni indeks, mRNK, proteinsku ekspresiju endotelnih receptora (ETA i ETB) i histopatologiju. C. sativumom primenjen oralno (1 g/kg TM) doveo je do poboljšanja hemodinamskih parametara, obnovio je srčane antioksidacione enzime, snizio oksidacioni stres, poboljšao lipidni profil, smanjio aterogeni indeks, smanjio nivoe ETA i ETB receptora mRNA i proteina i obnovio srčanu morfologiju. Naši rezultati ukazuju da se C. sativum može koristiti kod srčane insuficijencije kao kardioprotektivni agens, najverovatnije zbog njegove sposobnosti da ublaži oksidacioni stres, poboljša lipidni profil i endotelnu disfunkciju.

Ključne reči: *korijander, oksidacioni stres, srčana insuficijencija, endotelna disfunkcija, endotelinski receptori*

ABBREVIATIONS

AC- Atherogenic Coefficient
AIP- Atherogenic Index of Plasma
C. sativum- Coriandrum sativum
DBP- Diastolic blood pressure
GPx- Glutathione peroxidase
GR- Glutathione reductase
GSH- Glutathione
HDL-C- High-density lipoprotein-cholesterol
HF- Heart failure
HR- Heart rate
MAP- Mean arterial pressure
ISO- Isoproterenol
LDL- Low-density lipoproteincholesterol
MDA- Malondialdehyde
ROS- Reactive Oxygen species
SBP- Systolic blood pressure
SOD- Superoxide dismutase
TBA- Thiobarbituric acid
TC- Total cholesterol
TCA- Trichloroacetic acid
TG- Triglycerides;
vLDL-C-Very low-density lipoprotein-cholesterol

UDK: 615.322:582.794.1; 616.12-008.46-085.322
Ser J Exp Clin Res 2018; 19 (2): 119-129;
DOI: 10.1515/SJECR-2017-0080

Corresponding author: Adila Parveen
Centre for Physiotherapy and Rehabilitation Sciences, Jamia Millia Islamia, Jamia Nagar, New Delhi-110025, India, Telephone :+918587868142 email: adilaprvn@gmail.com

INTRODUCTION

Heart failure (HF) is a clinical syndrome, caused due to structural and functional cardiac abnormalities, characterized by changes in the hemodynamic and neurohumoral mechanisms that result in inability of the heart to pump blood efficiently. Epidemiological studies suggest high prevalence of cardiovascular diseases out of which heart failure accounts for more than 23 million cases worldwide (1). Major risk factors include ischemic heart disease, hypertension, coronary artery disease, cardiomyopathies, valvular and congenital heart disease, arrhythmias, pericardial disease, and cardiotoxic substances (2). Despite many technological advances in the field of cardiovascular diseases prevention, the incidence and prevalence of HF continue to increase (3).

In the recent years, considerable progress has been made to understand the mechanisms underlying heart failure. Evidence suggests the role of reactive oxygen species (ROS) in the pathogenesis of cardiovascular diseases (4). Increased production of ROS causes peroxidation of cell membrane lipids resulting in cardiac oxidative stress. Lipids are known to regulate cardiac function and altered lipid profile or high cholesterol levels are established predictors of atherogenesis and cardiovascular mortality (5, 6). Reduced oxidative stress and cholesterol levels has been associated with cardioprotection mechanisms (7). Vascular endothelin receptors are a class of G-protein coupled receptors that mediate vascular smooth muscle contraction and are known to play an important role in cardiovascular system regulation (8). Several experimental studies have suggested the role of ET_A and ET_B receptor systems to be highly dysregulated in chronic heart failure (8, 9).

Various treatment modalities for heart failure are available which include aldosterone inhibitors, angiotensin converting enzyme (ACE) inhibitors, angiotensin II receptor blockers (ARBs), beta-blockers, diuretics, calcium channel blockers and digoxin. Statins or 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors is a standard drug used for the treatment of heart failure by the virtue of its lipid lowering properties and hence has shown to reduce the incidence and mortality rate in HF patients (10). Many pleiotropic properties of statins beyond cholesterol reduction include its antioxidant, anti-inflammatory properties, improvement in endothelial dysfunction, release of endothelial progenitor cells, and a number of anti-tumor activities (11, 12). Despite many beneficial effects, statins have been reported to cause many adverse effects in the long term (13) which limits the use of statins to a very narrow range. Hence, the need to find an alternate strategy for the prevention and treatment of heart failure is very important.

Investigations are being carried out to study the role of herbal products for the treatment of various diseases as they are considered safe with minimal or no side effects. *C. sativum,* an important spice crop is mainly used in flavouring foods and has also been used as a medicinal plant for the treatment of various diseases (14). *C. sativum* is known to possess hypolipidemic (15), hypocholesterolemic (16) and antioxidant (17) properties. A study conducted in rats has also shown the cardiopotective action of *C. sativum* against myocardial necrosis (18).

Till date, no work has been done regarding the cardioprotective aspects of *C. sativum* in HF model. Therefore, in the light of above inferences, we designed this study to evaluate the therapeutic and prophylactic potential of *C. sativum* on hemodynamic changes, oxidative stress, lipid profile and endothelin receptors, in comparision with simvastatin against isoproterenol induced HF in wistar rats.

MATERIALS AND METHODS

Animals

Studies were performed on male Wistar rats (150–200 g, 6–8 weeks old). All experiments were conducted in compliance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and were approved by the Institutional Animal Ethics Committee. All rats had access to water and rodent chow ad libitum.

Coriandrum sativum seed extract

Dried coriander seeds (*C. sativum*) were powdered and mixed with distilled water to make aqueous suspension. It was administered to rats orally at a dose of 1g/kg b.wt. in accordance with Lal et al. (15)

Induction of heart failure (HF) and experimental groups

HF was induced by subcutaneous injections of isoproterenol (85 mg/kg) dissolved in normal saline, daily for two consecutive days at an interval of 24h. These animals were kept for 15 days for the development of HF model (19). Animals were randomly divided into 8 groups with 7 rats in each. Group I (Control): Control rats were fed normal pellet diet. Group II (HF): Rats were given ISO (85 mg/kg, subcutaneously) once at an interval of 24 h for two consecutive days and were kept for 15 days. Group III: (Therapeutic simvastatin): Rats were treated as in group II, in addition they received simvastatin (10 mg/kg; oral gavage) for another 15 days. Group IV (Therapeutic *C. sativum*): Rats were treated as in group II, in addition they received *C. sativum* 1g/kg; oral gavage) for another 15 days. Group V (Prophylactic simvastatin): Rats were pretreated with simvastatin (10 mg/kg; oral gavage) for 15 days and at the 15th day subcutaneously injected with ISO (85 mg/kg) for 2 consecutive days and were continued with simvastatin treatment for another 15 days. Group VI (Prophylactic *C. sativum.*): Rats were pretreated with *C. sativum* (1g/kg; oral gavage) for 15 days and at the 15th day subcutaneously injected with ISO (85 mg/kg) for two consecutive days and were continued with coriander treatment for another 15 days. Group VII (Simvastatin): Rats were treated with simvastatin (10 mg/kg; oral gavage) for 15 days. Group VIII (*C. sativum*): Rats were treated with *C. sativum.* (1 g/kg; oral gavage) for 15 days.

Hemodynamic Measurements

Rats were anaesthetized with intraperitoneal injection of urethane at a dose of 1 gm/kg b.wt. Disappearance of pedal reflexes indicated adequate anesthesia. Body temperature of the rat was maintained at 37°C to 38°C. Tracheostomy was performed to allow free air breathing without any obstruction. Femoral artery was cannulated by a polyethylene catheter filled with heparin solution (500 IU/mL, v/v), for recording arterial blood pressure (ABP). The catheter was attached to a 23-gauge needle connected through a three way stopcock to a pressure transducer (Statham-P23D, Oxnard, California). Femoral vein of the other limb was cannulated for injecting drugs. Before recording ABP, the catheter was flushed with heparinized saline solution (500 IU/mL, v/v) to prevent the formation of any blood clot that might interfere with the normal recording of ABP. The pressure recording system was calibrated with a mercury manometer before each experiment. ABP was measured after 20 minutes of stabilization. SBP, DBP, MAP, and HR were recorded on Power Lab data-acquisition system (4SP, AD Instruments, Australia) with a computerized analysis program (Lab Chart 7, AD Instruments).

Post-mitochondrial supernatant preparation and biochemical estimations

Left ventricle was removed quickly, cleaned of extraneous material and immediately perfused with ice-cold saline (0·85% NaCl) and were then homogenised in chilled phosphate buffer (0·1 M, pH 7·4) containing KCl (1·17%) using a Potter–Elvehjen homogeniser. The homogenate was filtered through muslin cloth and centrifuged at 800 g for 5 min at 4°C in a REMI cooling centrifuge to separate the nuclear debris. Aliquot obtained was centrifuged at 12,000 rpm for 20 min at 4°C to obtain the PMS, which was used as a source of enzymes. All biochemical estimations were completed within 24 h of animal sacrifice.

Estimation of glutathione

Glutathione (GSH) level was assessed by the method of Jollow et al. (20). A quantity of 1 ml of 10% PMS mixed with 1 ml of 4% sulphosalicylic acid, incubated at 4°C for 1 h, and then centrifuged at 4°C at 1200 g for 15 min. The reaction mixture of 3 ml was composed of 0·4 ml of supernatant, 2·2 ml phosphate buffer (0·1 M, pH 7·4) and 0·4 ml dithio-bis-2-nitrobenzoic acid (4 mg/ml). The yellow colour developed was read immediately at 412 nm on the spectrophotometer (Lambda EZ201; Perkin Elmer). GSH concentration was calculated as µmol GSH conjugates/ mg tissue.

Assay for glutathione peroxidase activity

Activity of Glutathione peroxidase (GPx) was calculated by the method of Mohandas et al. (21). The total volume of 2 ml was composed of 0·1 ml EDTA (1mM), 0·1 ml sodium azide (1mM), 1·44 ml phosphate buffer (0·1 M, pH 7·4), 0·05 ml glutathione reductase (1 IU/ml is equivalent to 1 mol GSSG reduced/min per ml), 0·05 ml GSH (1mM), 0·1 ml NADPH (0·2mM), 0·01 ml H_2O_2 (0·25mM) and 0·1 ml of 10% PMS. The depletion of NADPH at 340nm was recorded at 25°C. Enzyme activity was calculated as µmol NADPH oxidised/ min per mg protein with a molar extinction coefficient of $6{\cdot}22 \times 10^3\ M^{-1}\ cm^{-1}$.

Glutathione reductase activity

GR activity was determined by the method of Carlberg and Mannervik (22). The reaction mixture consisted of 1·65 ml phosphate buffer (0·1 M, pH 7·6), 0·1 ml EDTA (0·5mM), 0·05 ml GSH (1mM), 0·1 ml NADPH (0·1mM) and 0·1 ml of 10% PMS, in a total volume of 2 ml. Enzyme activity was quantified at 25°C by measuring the disappearance of NADPH at 340 nm and was calculated as µmol NADPH oxidized/min per mg protein using a molar extinction coefficient of $6{\cdot}22 \times 10^3\ M^{-1}\ cm^{-1}$.

Assay for catalase activity

Catalase activity was assessed by the method of Claiborne (23). In short, the reaction mixture consisted of 0·05 ml PMS, 1·0 ml of H_2O_2 (0·019 M), 1·95 ml phosphate buffer (0·1 M, pH 7·4), in a total volume of 3 ml. Changes in absorbance were recorded at 240 nm, and the change in absorbance was calculated as µmol H_2O_2 consumed/min per mg protein.

Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured by the method of Marklund and Marklund (24). The reaction mixture consisted of 2·875 ml Tris–HCl buffer (50mM, pH 8·5), pyrogallol (24mM in 10mM-HCl) and 100 ml PMS, in a total volume of 3 ml. Enzyme activity was measured at 420nm and was expressed as units/mg protein. One unit of enzyme is defined as the enzyme activity that inhibits the autooxidation of pyrogallol by 50%.

Measurement of lipid peroxidation (LPO)

Assay for lipid peroxidation (LPO) was done according to the method of Wright et al. (25). The reaction mixture consisted of 0.05 ml serum sample, 0.73 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml ascorbic acid (100mM) and 0.02 ml ferric chloride (100mM) in a total volume of 1 ml. This mixture was then incubated at 37°C in a shaking water bath for 1 h. Reaction was stopped by the addition of 1 ml trichloroacetic acid (10%). Following the addition of 1 ml thiobarbituric acid (TBA) (0·67%), all the tubes were placed in a boiling water bath for 20 min. The tubes were shifted to an ice bath, and then centrifuged at 2500 g for 10 min. Amount of malondialdehyde (MDA) formed in the serum samples was assessed by measuring the optical density of the supernatant at 532 nm against blank using a molar extinction coefficient of $1{\cdot}56 \times 10^5 M^{-1} cm^{-1}$.

Lipid profile

Serum total cholesterol (TC), triglycerides (TG), low-density lipoproteincholesterol (LDL-C), very low-density lipoprotein-cholesterol (VLDL-C) and high-density lipopro-

tein-cholesterol (HDL-C) were determined by commercially available spectrophotometric assay kits (Monozymes, India).

Atherogenic indices

Atherogenic indices were calculated as described by Ikewuchi and Ikewuchi (26) using the formulae:

Cardiac Risk Ratio (CRR) = TC / HDL-C
Atherogenic Coefficient (AC) = (TC–HDL-C) / HDL-C
Atherogenic Index of Plasma (AIP) = Log (TG / HDL-C)

(The values of TC, TG and HDL-C were converted to mmol/l for calculation of atherogenic indices).

RNA isolation and quantitative real-time reverse transcription-polymerase chain reaction

Total tissue RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction method using Trizol reagent (Invitrogen, USA) following the manufacturer's instructions. The tissue was homogenized in trizol (0.2 g tissue per 2 ml trizol) with a Polytron tissue homogenizer. Chloroform extraction, isopropanol precipitation, and 75% (vol/vol) ethanol washing of precipitated RNA were subsequently performed. The obtained RNA was resolved in diethyl pyrocarbonate (DEPC) treated water. Quantity and quality of RNA extracted was analyzed by NanoDrop (ND-3300, NanoDrop Technologies, USA) and agarose gel electrophoresis. One microgram total RNA was reverse transcribed using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas Life Sciences, USA) as per the manufacturer's recommendations.

Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed using the Light cycler SYBR Green RT-PCR kit (Roche, U.S.A) with the help of specific primers for endothelin receptor A (ET_A) and endothelin receptor B (ET_B), and normalized to Glyceraldehydes 3-phosphate dehydrogenate (GAPDH) following the standard protocol. Analysis was performed using $\Delta\Delta C_t$ method as described earlier (27). The sequence of the primers used is shown in Table 1.

Western blotting

Total protein was extracted from the heart left ventricle. Protein concentration was determined by Bradford assay. Denaturation of proteins was performed in 2X Laemmli sample buffer by heating to 95°C for 5 min, followed by a quick spin. Total protein (40 µg) in each sample was separated by 10% SDS polyacrylamide gel electrophoresis and transferred to methanol-activated PVDF membrane in Tris-Glycine buffer containing 20% of methanol. Membranes were blocked by TBST with BSA and incubated with rat polyclonal antiserum raised against rat ET_AR (1: 200) and rat ET_BR (1: 100), followed by incubation with horseradish peroxidase conjugated secondary antibody. β-actin was used as an internal control. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection system. Densitometric analysis was performed using image-analysis software to determine the protein level. The band intensities of target proteins were expressed relative to β-actin and normalized to the percentage of control.

Table 1: Primer sequences of ET_A receptor, ET_B receptor and GAPDH genes for Real Time PCR.

Gene	Primer Sequence
ET_A receptor (sense)	5′-ATCGCTGACAATGCTGAGAG-3′
ET_A receptor (antisense)	5′-CCACGATGAAAATGGTACAG-3′
ET_B receptor (sense)	5′-GAAAAGAGGATTCCCACCTG-3′
ET_B receptor (antisense)	5′-ACGAACACGAGGCATGATAC-3′
GAPDH (sense)	5′-GCCATCAACGACCCCTTCATTG-3′
GAPDH (antisense)	5′-TGCCAGTGAGCTTCCCGTTC-3′

Statistical Analysis

The results were presented as mean ± standard error of the mean (SEM). All the data was analyzed by analysis of variance (ANOVA) followed by Tukey multiple comparison tests, for the analysis between the groups. The minimum criterion for statistical significance was set at $P < 0.05$ for all comparisons.

Histopathological Examination

The heart (left ventricle) was fixed in 10% formalin solution for histopathological analysis. The sections were examined under light microscope, and photomicrographs were taken. Tissues were embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (H&E). The sections were examined under light microscope, and photomicrographs were taken.

RESULTS

Arterial blood pressure and Heart rate

HF rats showed a significant ($p<0.05$) decrease in SBP, DBP, HR and MAP as compared to the control group. However, both therapeutic and prophylactic treatment with *C. sativum* and simvastatin significantly ($p<0.05$) increased SBP, DBP, HR and MAP compared to the HF group. No significant changes were observed in *C. sativum* and simvastatin only treated groups. The results are as shown in Figure 1.

Enzymatic and non-enzymatic antioxidant levels

Table 2 demonstrates the activities of different antioxidant enzymes in response to *C. sativum* and simvastatin treatment in ISO induced rats. ISO administration significantly reduced cardiac GSH ($p<0.05$) levels in HF rats as compared to the control rats. Activities of GR ($p<0.05$), GPx ($p<0.05$), SOD ($p<0.05$) and catalase ($p<0.05$) were also found to be decreased in ISO induced rats when compared with the control group. Therapeutic and prophylactic treat-

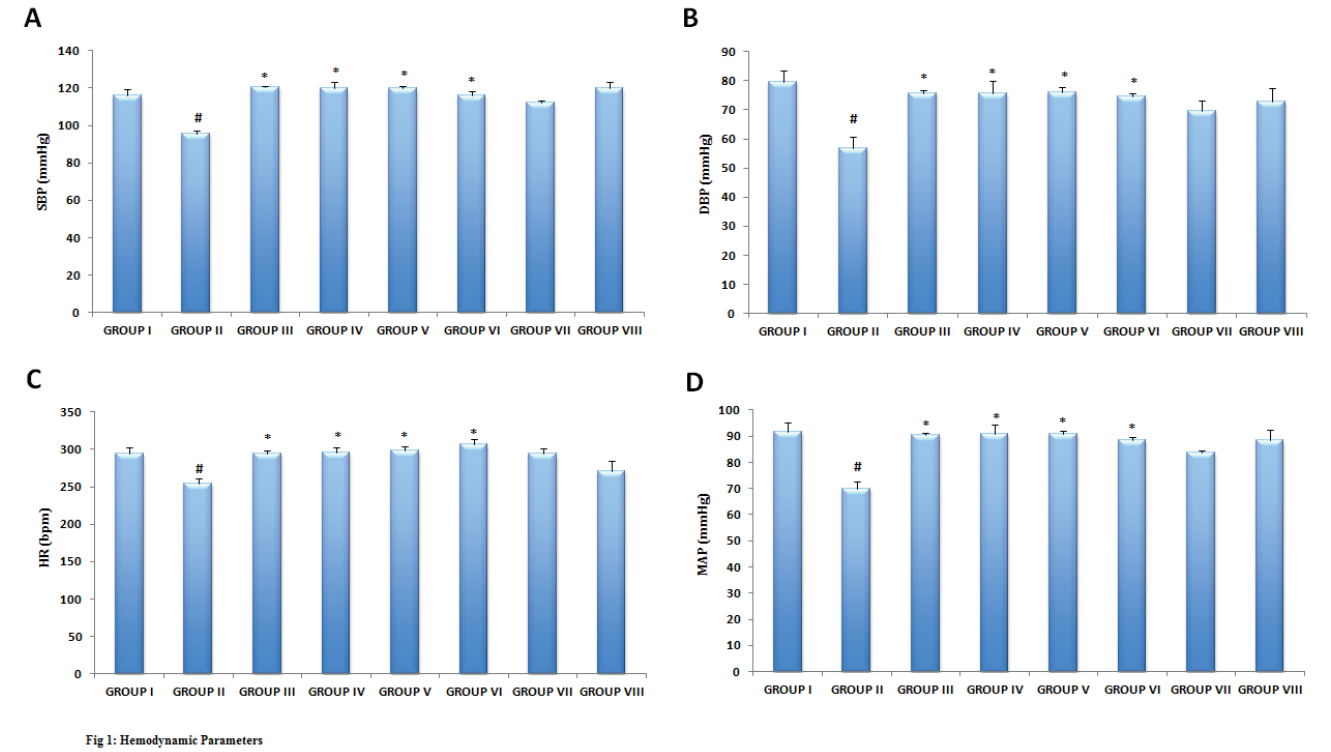


Fig 1: Hemodynamic Parameters

Figure 1: Hemodynamic parameters: **(A)** Systolic blood pressure (SBP), **(B)** Diastolic blood pressure (DBP), **(C)** Heart rate (HR), **(D)** Mean arterial pressure in Group I (Control); Group II (HF); Group III (Therapeutic simvastatin); Group IV (Therapeutic *C. sativum*); Group V (Prophylactic simvastatin); Group VI (Prophylactic *C. sativum*); Group VII (Simvastatin per se); Group VIII (*C. sativum* per se). Results represent mean ± SEM of seven animals per group. Results obtained are significantly different from control group (#p< 0.05). Results obtained are significantly different from HF group (*p < 0.05).

ment with *C. sativum* and simvastatin however increased the level of GSH (p<0.05) and activities of GR (p<0.05), GPx (p<0.05), SOD (p<0.05) and catalase (p<0.05), compared to the HF group. However, no significant difference was found in the *C. sativum* and simvastatin only treated group, when compared to the control group.

Lipid peroxidation

In comparision with the control group, levels of MDA increased significantly (p<0.05) in HF rats. Administration of *C. sativum* and simvastatin both therapeutically and prophylactically showed significant (p<0.05) reduction in MDA levels when compared with the HF group. *C. sati-*

Table 2: Effect of simvastatin and *Coriandrum sativum* treatment on cardiac glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase levels in isoproterenol induced rats.

Treatment groups	GSH (µmol GSH/g tissue)	GPx (µmol NADPH oxidized/min/mg protein)	GR (µmol NADPH oxidised/min/mg protein)	Catalase (µmol H_2O_2 consumed/min/mg protein)	SOD (units/mg protein)
Group I	4.24 ± 0.32	7.20 ± 0.72	9.18 ± 0.58	88.40 ± 2.40	3.90 ± 0.38
Group II	1.77 ± 0.14#	3.52 ± 0.24#	4.50 ± 0.42#	42.23 ± 1.98#	1.52 ± 0.22#
Group III	3.25 ± 0.20*	5.19 ± 0.33*	6.54 ± 0.24*	67.18 ± 3.20*	2.69 ± 0.20*
Group IV	3.28 ± 0.25*	5.28 ± 0.27*	6.62 ± 0.57*	68.12 ± 2.33*	2.72 ± 0.24*
Group V	3.46 ± 0.19*	6.29 ± 0.32*	8.53 ± 0.54*	71.97 ± 2.44*	3.52 ± 0.18*
Group VI	3.48 ± 0.28*	6.37 ± 0.30*	8.62 ± 0.32*	72.23 ± 2.09*	3.57 ± 0.15*
Group VII	4.22 ± 0.31	7.23 ± 0.42	9.20 ± 0.44	88.57 ± 1.11	3.92 ± 0.24
Group VIII	4.25 ± 0.29	7.28 ± 0.50	9.23 ± 0.51	88.73 ± 2.78	3.94 ± 0.21

Results represent mean ± SEM of seven animals per group. Group I (Control); Group II (HF); Group III (Therapeutic simvastatin); Group IV (Therapeutic *C. sativum*); Group V (Prophylactic simvastatin); Group VI (Prophylactic *C. sativum*.); Group VII (Simvastatin per se); Group VIII (*C. sativum* per se). Results obtained are significantly different from control group (#p< 0.05). Results obtained are significantly different from HF group (*p < 0.05).

Table 3: Effect of simvastatin and *coriandrum sativum* L. treatment on lipid profile of isoproterenol induced rats.

Treatment groups	Cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	vLDL (mg/dl)
Group I	118.45 ± 1.48	93.18 ± 3.10	47.18 ± 0.82	42.16 ± 0.73	21.30 ± 0.84
Group II	164.1 ± 2.43$^{\#}$	163.33 ± 3.73$^{\#}$	22.5 ± 0.34$^{\#}$	81.25 ± 3.42$^{\#}$	29.56 ± 0.89$^{\#}$
Group III	148.73 ± 4.05*	146.63 ± 2.80*	30.33 ± 0.98*	71.43 ± 2.13*	25.43 ± 0.91*
Group IV	150.66 ± 1.87*	143.85 ± 3.71*	28.5 ± 1.78*	72.46 ± 2.05*	25.85 ± 0.46*
Group V	132.88 ± 1.86*	111.4 ± 2.0*	43.1 ± 0.84*	50.06 ± 1.2*	23.1 ± 0.45*
Group VI	133.33 ± 2.60*	109.03 ± 2.63*	42.33 ± 0.55*	51.36 ± 0.95*	23.28 ± 0.32*
Group VII	116.91 ± 1.46	90.7 ± 2.79	48.3 ± 0.67	40.36 ± 0.4	20.33 ± 0.38
Group VIII	117.56 ± 0.60	91.61 ± 3.28	47.96 ± 0.71	41.93 ± 0.62	21.0 ± 0.64

Results represent mean ± SEM of seven animals per group. Group I (Control); Group II (HF); Group III (Therapeutic simvastatin); Group IV (Therapeutic *C. sativum*.); Group V (Prophylactic simvastatin); Group VI (Prophylactic *C. sativum*); Group VII (Simvastatin per se); Group VIII (*C. sativum* per se). Results obtained are significantly different from control group (#p< 0.05). Results obtained are significantly different from HF group (*p < 0.05).

vum and simvastatin alone treated groups did not have any significant effect on the serum MDA levels (Figure 2).

Lipid profile

HF rats showed a significant (p<0.05) elevation in TC, TG, LDL-C, vLDL-C levels and a significant (p<0.05) reduction in the level of HDL-C respectively, compared to control rats. Therapeutic and prophylactic treatment with *C. sativum* and simvastatin significantly (p<0.05) decreased the levels of TC, TG, LDL-C, vLDL-C and increased (p<0.05) HDL-C levels when compared to HF rats. Treatment with *C. sativum* and simvastatin alone did not show any significant effect on the lipid profile (Table 3).

Atherogenic indices

Atherogenic indices were assessed by calculating Cardiac risk ratio (CRR), Atherogenic coefficient (AC) and Atherogenic index of plasma (AIP). A significant (p<0.05) increase in CRR, AC and AIP was observed in HF rats. However, therapeutic and prophylactic treatment with *C. sativum* and simvastatin showed a significant (p<0.05) reduction in CRR, AC and AIP. *C. sativum* and simvastatin per se treatment showed no significant changes in atherogenic indices when compared with the control group (Table 4).

Expression of ET_A and ET_B receptor mRNA levels

Quantitative real-time PCR was used to measure the mRNA levels of ET_A and ET_B receptors. Relative mRNA levels of ET_A receptors in the left ventricle of HF rats were found to be increased significantly (p<0.05) as compared with the control group. Both therapeutic and prophylactic treatment with *C. sativum* and simvastatin showed a significant (p<0.05) decrease in the levels of ET_A receptor mRNA, when compared to the HF group (Figure 3A). Further, mRNA levels of ET_B receptor were also found to be significantly (p<0.05) upregulated in HF group, compared to the normal control group. However, in comparision with the HF group therapeutic and prophylactic treatment with *C. sativum* and simvastatin significantly (p<0.05) downregulated the expression of ET_B receptor mRNA (Figure 3B). No significant change was observed

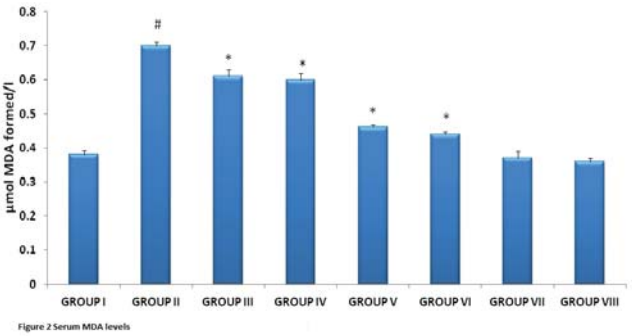


Figure 2 Serum MDA levels

Figure 2: Serum Malodialdehyde (MDA) levels. Results represent mean ± SEM of seven animals per group. Group I (Control); Group II (HF); Group III (Therapeutic simvastatin); Group IV (Therapeutic *C. sativum*); Group V (Prophylactic simvastatin); Group VI (Prophylactic *C. sativum*); Group VII (Simvastatin per se); Group VIII (*C. sativum* per se). Results obtained are significantly different from control group (#p< 0.05). Results obtained are significantly different from HF group (*p < 0.05).

Table 4: Effect of simvastatin and *C. sativum* treatment on Atherogenic Indices of isoproterenol induced rats.

Treatment groups	Cardiac Risk Ratio (CCR)	Atherogenic Coefficient (AC)	Atherogenic Index of Plasma (AIP)
Group I	2.51 ± 0.03	1.51 ± 0.03	-0.06 ± 0.016
Group II	7.30 ± 0.17$^{\#}$	6.30 ± 0.17$^{\#}$	0.50 ± 0.008$^{\#}$
Group III	4.92 ± 0.15*	3.92 ± 0.15*	0.32 ± 0.017*
Group IV	5.41 ± 0.42*	4.41 ± 0.42*	0.34 ± 0.023*
Group V	3.08 ± 0.07*	2.08 ± 0.07*	0.053 ± 0.013*
Group VI	3.14 ± 0.04*	2.14 ± 0.04*	0.052 ± 0.004*
Group VII	2.37 ± 0.04	1.37 ± 0.04	-0.09 ± 0.014
Group VIII	2.45 ± 0.04	1.45 ± 0.04	-0.07 ± 0.015

Results represent mean ± SEM of seven animals per group. Group I (Control); Group II (HF); Group III (Therapeutic simvastatin); Group IV (Therapeutic *C. sativum*); Group V (Prophylactic simvastatin); Group VI (Prophylactic *C. sativum*); Group VII (Simvastatin per se); Group VIII (*C. sativum* per se). Results obtained are significantly different from control group (#p< 0.05). Results obtained are significantly different from HF group (*p < 0.05).

in the ET_A and ET_B receptor mRNA levels of *C. sativum* and simvastatin alone treated groups.

Expression of ET_A and ET_B receptor protein levels

Protein levels of ET_A and ET_B receptor was evaluated by western blotting. Expression level of ET_A receptor protein was found to be significantly ($p<0.05$) upregulated in HF rats, when compared to the untreated control rats. However, in comparison with the HF rats, treatment with *C. sativum* and simvastatin both therapeutically and prophylactically reduced their levels significantly ($p<0.05$) as observed in Figure 4A. Levels of ET_B receptor protein was also found to be increased significantly ($p<0.05$), in comparison with the control group. Therapeutic treatment decreased the levels of ET_B receptor protein significantly ($p<0.05$) in *C. sativum* and simvastatin groups accompanied by further downregulation of ET_B receptor protein in prophylactic *C. sativum* and simvastatin groups significantly ($p<0.05$). The results are shown in Figure 4B. No significant change was assessed in the ET_A and ET_B receptor protein levels of *C. sativum* and simvastatin alone treated groups, when compared with the normal group.

Histopathology

Histological sections from left ventricle of control rats showed normal morphology. However, HF rats showed

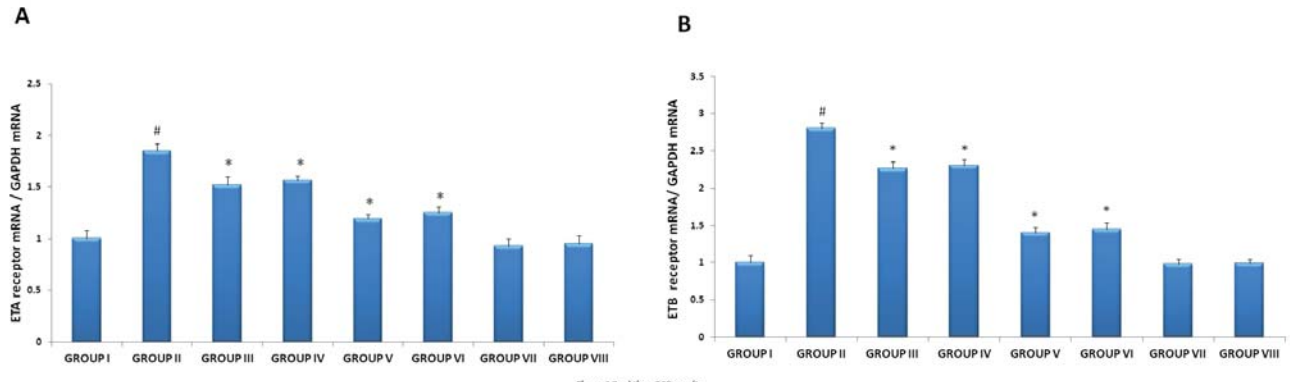


Figure 3: Real Time PCR results of ET_A and ET_B receptor mRNA in the LV **(A)** Relative mRNA levels of ET_A receptor, **(B)** Relative mRNA levels of ET_B receptor. Results represent mean ± SEM of seven animals per group. Group I (Control); Group II (HF); Group III (Therapeutic simvastatin); Group IV (Therapeutic *C. sativum*); Group V (Prophylactic simvastatin); Group VI (Prophylactic *C. sativum*); Group VII (Simvastatin per se); Group VIII (*C. sativum* per se). Results obtained are significantly different from control group (#p< 0.05). Results obtained are significantly different from HF group

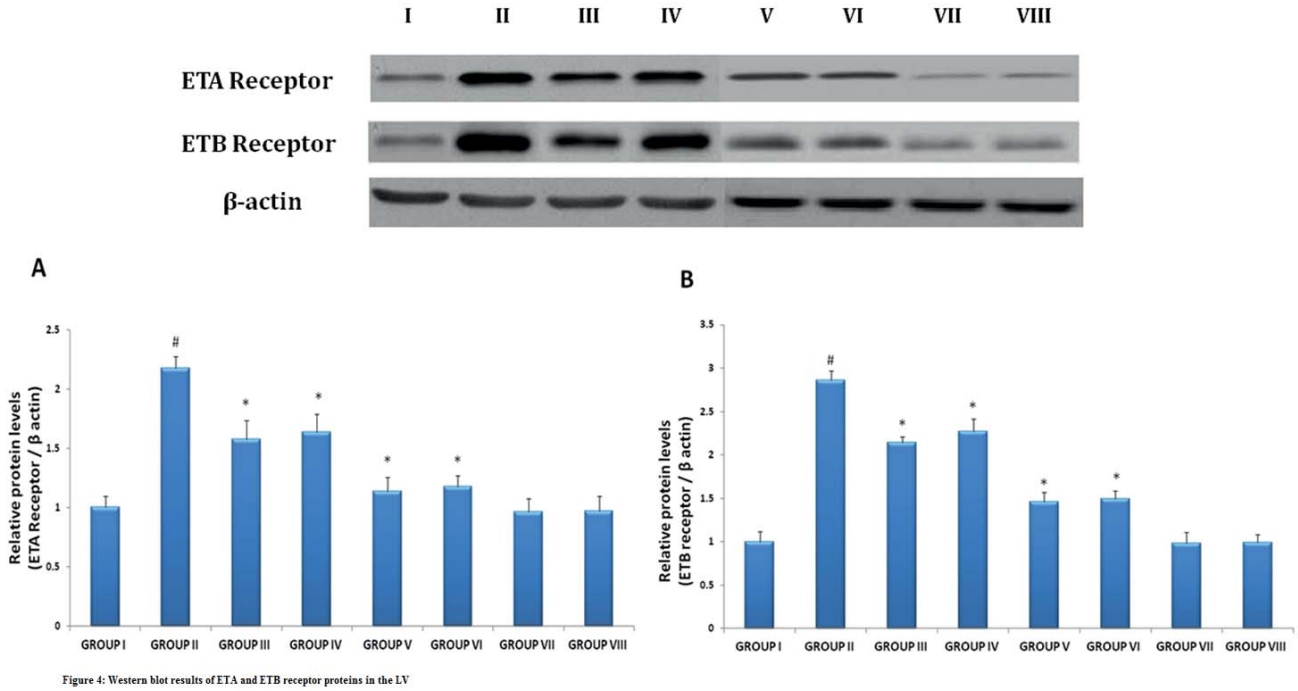


Figure 4: Western blot results of ETA and ETB receptor proteins in the LV

Figure 4: Western blot results of ET_A and ET_B receptor proteins in the LV. **(A)** ET_A receptor protein expression level, **(B)** ET_B receptor protein expression level. Results represent mean ± SEM of seven animals per group. Group I (Control); Group II (HF); Group III (Therapeutic simvastatin); Group IV (Therapeutic *C. sativum*); Group V (Prophylactic simvastatin); Group VI (Prophylactic *C. sativum*); Group VII (Simvastatin per se); Group VIII (*C. sativum* per se). Results obtained are significantly different from control group (#p< 0.05). Results obtained are significantly different from HF group (*p < 0.05).

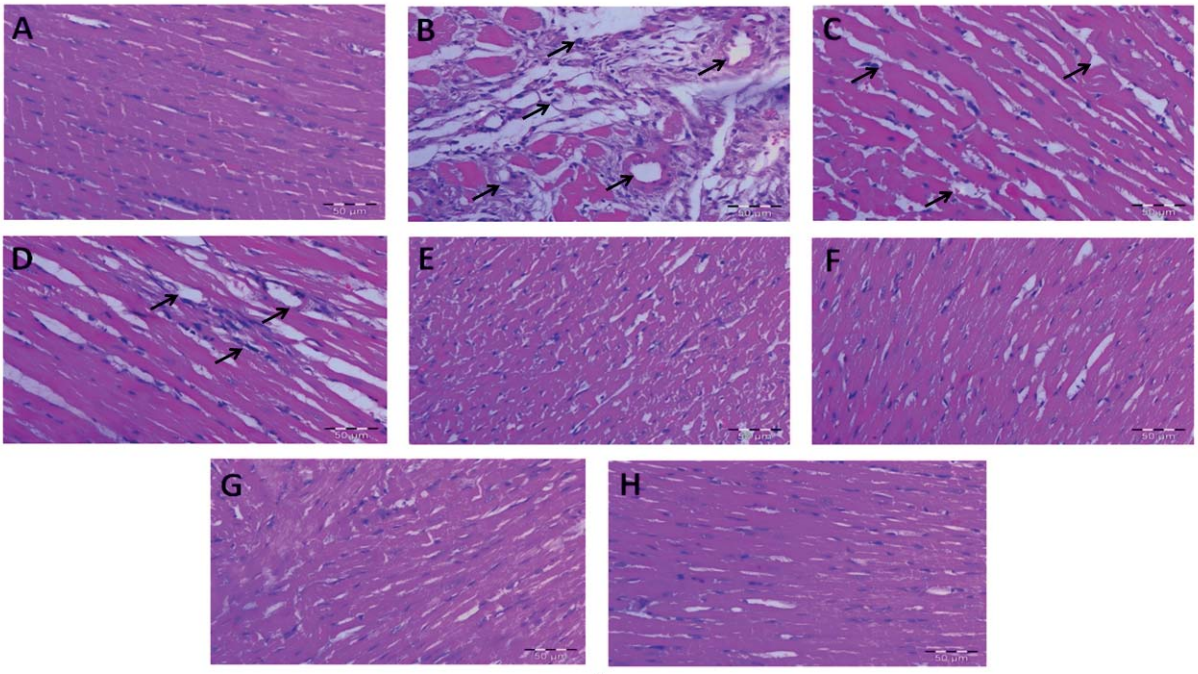


Figure 5: Histopathological changes in the myocardium

Figure 5: Histopathological changes in the myocardium (A) Control group showing normal architecture of cardiac myofibers (B) HF group showing alteration in myocardial structure with interstitial oedema, myocyte necrosis and inflammatory cell infiltration (C) Therapeutic simvastatin (D) Therapeutic *C. sativum* showing mild necrosis and inflammatory cell infiltration (E) Prophylactic simvastatin (F) Prophylactic *C. sativum* showing slight myocardial degeneration (G) Simvastatin per se (H) *C. sativum* per se. Specimens stained with hematoxylin and eosin.

distortion in the myocardial structure, myocyte necrosis with interstitial oedema and leucocyte infiltration. Therapeutic treatment with *C. sativum* and simvastatin showed partial improvement in myocardial morphology. However, prophylactic *C. sativum* and simvastatin treated groups showed marked improvement in the myocyte necrosis, interstitial oedema and leucocyte infiltration thereby restoring the normal myocardial morphology. No change in the myocardial architecture was observed from the histopathological sections of *C. sativum* and simvastatin alone treated groups (Figure 5).

DISCUSSION

The results of our present study clearly demonstrate the cardioprotective effect of *Coriandrum sativum* in isoproterenol induced HF rats. Isoproterenol (ISO), a synthetic catecholamine and a β-adrenergic receptor agonist induces myocardial necrosis leading to left ventricular hypertrophy. Mechanisms underlying ISO induced heart failure include oxidative stress due to excessive production of free radicals from oxidative metabolism of catecholamines, which result in both structural and functional myocardial injury (28, 29). Moreover, calcium overload and hypoxia followed by coronary hypotension and myocardial hyperactivity may be the cause for morphological alterations that results in myocardial damage (30). ISO induced heart failure serves as an excellent experimental model to evaluate the cardioprotective efficacy of various herbal and synthetic compounds.

Statin, a hypolipidemic drug is used in the treatment of chronic heart failure due to its ability to lower cholesterol levels, improve endothelial function, prevent thrombus formation and modulate inflammatory responses (31). Despite a number of beneficial properties, statins have been reported to cause detrimental effects like muscle toxicity, myopathy, rhabdomyolysis and liver damage (13). Hence, the development of alternate therapeutics which are safe and cost-effective is a good approach for the future. In this regard, herbal and natural products are being studied at the molecular and cellular level to understand their mechanism of action. In the present study, statins were used to compare its cardioprotective actions with the herbal drug *C. sativum.*

Pharmacological analysis of *C. sativum* seeds have revealed the presence of polyphenols (rutin, chlorogenic acid and caffeic acid derivatives), flavonoids (quercetin, isoquercetin, keampferol, rhamnetin and apigenin) and β-carotenoids (32, 33). The oil of *Coriandrum sativum* seeds is rich in α-pinene, β-pinene, camphor, citronellol, p-cymene, geraniol, limonene, linalool, myrcene, α-phellandrene, terpinene, monoterpenoid glycosides and their derivatives (33). Many of these compounds are known to prevent oxidative stress by inhibiting free radical generation in the cellular system, when obtained through diet (33). Flavonoids are known for their anti-inflammatory, antitumor and antioxidant activities (34). Hence, these constituents in combination or independently may be responsible for the cardioprotective properties of *C. sativum.*

Hemodynamic changes have been reported in cardiovascular diseases (35, 36). Our present study showed

significantly lower SBP, DBP, HR and MAP in HF rats as reported earlier (37). Therapeutic and prophylactic treatment with *C. sativum* and simvastatin, however attenuated the decline in SBP, DBP, HR and MAP, thus showing an improvement in altered hemodynamic profile.

Oxidative stress has been well implicated in the pathophysiology of cardiac remodeling and progression of heart failure (37). Increased production of reactive oxygen species damages the cell membrane lipids and causes lipid peroxidation. MDA is one of the final products of lipid peroxidation and a reliable marker for the assessment of oxidative stress (38). The enzymatic antioxidants are mainly involved in scavenging free radicals and reactive oxygen species. Previous studies have demonstrated that isoproterenol administration increases oxidative stress and depletes the level of cardiac GSH and antioxidant armory in the heart (39, 40). Our present study also suggested increased MDA formation and reduced GSH, GPx, GST, GR, catalase and SOD levels in HF rats. *C. sativum* treatment reduced MDA levels and improved the cardiac GSH content and antioxidant enzyme activities as reported previously (41) suggesting its free radical scavenging properties against cardiac oxidative stress in HF rats.

Lipids are known to play an important role in heart failure. Lipids provide structure and stability to cell membranes and changes in lipid profile may contribute to cell death resulting in myocardial ischemia. Altered lipid profile has been well established in cardiovascular diseases (42). Our present study demonstrated a significant increase in serum LDL, vLDL, total cholesterol, triglycerides and decrease in HDL levels in HF rats as reported previously (19). The mechanism of action of isoproterenol on fat cells is believed to be mediated by the cAMP cascade, where isoproterenol activates adenylate cyclase, thereby increasing the cAMP formation. Subsequently, cAMP promotes lipolytic activity by activating cAMP-dependent protein kinase that phosphorylates lipase resulting in the hydrolysis of stored triacylglycerol, which contributes to hyperlipidemia (43, 44). Therapeutic and prophylactic treatment with *C. sativum* however reduced the levels of total cholesterol, triglyceride, LDL, vLDL and increased HDL levels. Thus, C. sativum administration modulated the level of lipid and lipoproteins by decreasing lipid uptake and enhancing lipid breakdown suggesting the hypolipidemic property of *C. sativum* thus conferring cardioprotective effect. *C. sativum* has also known to inhibit the enzyme HMG CoA reductase which is the key enzyme in the pathway of cholesterol biosynthesis in liver (45). Simvastatin treatment in HF rats also showed a similar effect.

Atherogenic indices are powerful indicators of the risk of cardiovascular disease which is assessed by evaluating CRR, AC and AIP, the higher the value the higher the risk of developing heart disease. AIP has been reported to be a significant predictor of atherosclerosis (46). In our present study, HF rats showed a significant increase in CRR, AC and AIP. However, therapeutic treatment with *C. sativum* and simvastatin decreased CRR, AC and AIP which was further reduced by prophylactic treatment of *C. sativum* and simvastatin signifying their role in reducing the probability of cardiovascular pathogenesis.

Vascular endothelium has an important role in cardiovascular system regulation. Endothelin (ET-1) secreted from vascular endothelial cells is a potent vasoconstrictor, that has affinity for two types of receptors namely ET_A and ET_B receptor. Both these receptors co-exist on vascular smooth muscle cells (VSMC) that mediates vasoconstriction. In addition ET_B receptors present on vascular endothelial cells (VEC) contributes to vasodilatation and ET-1 clearance (47). It has been suggested that in cardiovascular diseases, vasodilator ET_B receptors may switch their phenotype to contractile ET_B receptors (48, 49) and that the increased expression of ET_B receptors is directly correlated with the degree of ischemic heart disease (50). In the present study mRNA levels of ET_A and ET_B receptors was found to increase significantly in HF rats as compared to the control rats followed by increased expression of ET_A and ET_B receptor protein levels. Our data is consistent with the earlier studies (9). Therapeutic and prophylactic treatment with *C. sativum* and simvastatin reduced ET_A and ET_B receptor mRNA levels as compared with the HF treated rats. Their protein levels were also found to be significantly lower as compared with the HF rats.

C. sativum cardioprotective efficacy was further supported by histopathological studies. Histological findings revealed distorted myocardial architecture, cardiac myocyte hypertrophy, fibrosis and myocardial necrosis in isoproterenol induced rats. Therapeutic and prophylactic treatment with *C. sativum* and simvastatin alleviated isoproterenol induced myocardial changes and left ventricular hypertrophy. Hence, our results provide strong evidence indicating the cardioprotective efficacy of *C. sativum* against isoproterenol induced heart failure.

In conclusion, *C. sativum* may serve as an alternative herbal drug in protection from heart failure. The cardioprotective action of *C. sativum* may be due to its ability to improve the hemodynamic profile, alleviate oxidative stress, lower cholesterol levels, improve endothelial dysfunction and restore the cardiac morphology.

ACKNOWLEDGMENTS

Authors would like thank Central Council for Research in Unani Medicine (CCRUM), ministry of health, Govt of India for providing funds to carry out this research work.

REFERENCES

1. Bui AL, Horwich TB, Fonarow GC. Epidemiology and risk profile of heart failure. Nat. Rev. Cardiol. 2011; 8:30–41.
2. Mosterd A, Hoes AW. Clinical epidemiology of heart failure. Heart. 2007; 93:1137–1146.
3. Ramani GV, Uber PA, Mehra MR. Chronic Heart Failure: Contemporary Diagnosis and Management. Mayo. Clin. Proc. 2010; 85:180–195.

4. Moris D, Spartalis M, Spartalis E, Karachaliou GS, Karaolanis GI, Tsourouflis G, Tsilimigras DI, Tzatzaki E, Theocharis S. The role of reactive oxygen species in the pathophysiology of cardiovascular diseases and the clinical significance of myocardial redox. Ann Transl Med. 2017; 5(16): 326.
5. Choy PC, Siow YL, Mymin DOK. Lipids and atherosclerosis. Biochem. Cell. Biol. 2004; 82:212-24.
6. Daniels TF, Killinger KM, Michal JJ, Wright RW Jr, Jiang Z. Lipoproteins, cholesterol homeostasis and cardiac health. Int J Biol Sci. 2009; 5(5): 474-88.
7. Olatunji LA, Soladoye AO. Effect of increased magnesium intake on plasma cholesterol, triglyceride and oxidative stress in alloxan-diabetic rats. Afr. J. Med. Med. Sci. 2007; 36:155-61.
8. Xu CB, Sun Y, Edvinsson L. Cardiovascular risk factors regulate the expression of vascular endothelin receptors. Pharmacol. Ther. 2010; 127:148-55.
9. Kobayashi T, Miyauchi T, Sakai S, et al. Expression of endothelin-1, ETA and ETB receptors, and ECE and distribution of endothelin-1 in failing rat heart. Am. J. Physiol. 1999; 276:H1197-206.
10. Liao JK, Laufs U. Pleiotropic effects of statins. Annu. Rev. Pharmacol. Toxicol. 005; 245:89–118.
11. Kavalipati N, Shah J, Ramakrishan A, Vasnawala H. Pleiotropic effects of statins Indian J Endocrinol Metab. 2015; 19(5): 554–562.
12. Haehling SV. Statins for heart failure: still caught in no man's land? Clinical Science. 2009; 116:37–39.
13. Gotto AM. Statins, cardiovascular disease and drug safety. Am. J. Cardiol. 2006; 97:3-5.
14. Maghrani M, Zeggwagh N, Haloui M, Eddouks M. Acute diuretic effect of aqueous extract of Retamaraetam in normal rats. J. Ethnopharmacol. 2005; 99:31-35.
15. Lal AAS, Tkumar PBM. Pillai KS. Hypolipidemic effect of Coriandrum sativum L. intriton-induced hyperlipidemic rats. Indian J. Exp. Biol. 2004; 42:909–912.
16. Dhanapakiam P, Joseph JM, Ramaswamy VK. The cholesterol lowering property of coriander seeds (Coriandrum sativum): Mechanism of action. J. Environ. Biol. 2008; 29:53-56.
17. Samojlik I, Lakic N, Mimica-Dukic N, Dakovic-Svajcer K, Bozin B. Antioxidant and hepatoprotective potential of essential oils of coriander (Coriandrum sativum L.) and caraway (Carum carvi L.) (Apiaceae). J. Agric. Food Chem. 2010; 56:139- 147.
18. Patel DK, Desai SN, Gandhi HP, Devkar RV, Ramachandran AV. Food and Chemical Toxicology. Cardio protective effect of Coriandrum sativum L. on isoproterenol induced myocardial necrosis in rats. Food Chem. Toxicol. 2012; 50:3120–3125.
19. Parveen A, Babbar R, Agarwal S, Kotwani A, Fahim M. Terminalia arjuna enhances baroreflex sensitivity and myocardial function in isoproterenol-induced chronic heart failure rats. J. Cardiovasc. Pharmacol. Ther. 2012; 17:199-207.
20. Jollow DJ, Mitchell JR, Zampaglione N, Gillete JR. Bromobenzene induced liver necrosis: protective role of Glutathione and evidence for 3-4,bromobenzene oxide as the hepatotoxic intermediate. Pharmacology 1974; 11:151-159.
21. Mohandas M, Marshall JJ, Duggin GG, Horvath JS, Tiller D. Differential Distribution Of Glutathione And Glutathione Related Enzymes In Rabbit Kidney. Cancer Research 1984; 44:5086–5091.
22. Carlberg L, Mannervick B. Glutathione reductase level in rat brain. Journal of biological chemistry 1975; 250: 5575-5580.
23. Clairborne A. Catalase activity, CRC Press, Handbook of Methods for Oxygen Radical Research; R. Greenwald, 1985; Ed: 283–284.
24. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur J Biochem 1974; 47:469–474.
25. Wright JR, Colby HD, Miles PR. Cytosolic factors which affect microsomal lipid peroxidation in lung and liver. Arch. Biochem. Biophys. 1981; 206:296–304.
26. Ikewuchi JC, Ikewuchi CC. Alteration of plasma lipid profile and atherogenic indices of cholesterol loaded rats by Tridax procumbensm Linn: Implications for the management of obesity and cardiovascular diseases. Biokemistri. 2009; 21:95-9.
27. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001; 29:2003-2007.
28. Khalil MI, Ahmmed I, Ahmed R, Tanvir EM, Afroz R, Paul S, Gan SH, Alam N. Amelioration of Isoproterenol-Induced Oxidative Damage in Rat Myocardium by Withania somnifera Leaf Extract. Biomed Res Int. 2015; 2015: 624159.
29. Sharma M, Kishore K, Gupta SK, Joshi S, Arya DS. Cardioprotective potential of ocimum sanctum in isoproterenol induced myocardial infarction in rats. Mol. Cell. Biochem. 2001; 225: 75-83.
30. Yeager JC, Iams SG. The hemodynamics of isoproterenol-induced cardiac failure in the rat. Circ. Shock. 1981; 8:151-63.
31. Dickinson MG, John H, Olshansky B, et al. Statin use was associated with reduced mortality in both ischemic and nonischemic cardiomyopathy and in patients with implantable defibrillators. Am. Heart. J. 2007; 153:573-578.
32. Melo EA, Bion FM, Filho JM, Guerra NB. In vivo antioxidant effect of aqueous and etheric coriander (Coriandrum sativum L.) extracts. Eur. J. Lipid Sci. Technol. 2003; 105:483-487.
33. Rajeshwari U, Andallu B. Medicinal benefits of coriander (Coriandrum Sativum L). Spatula DD. 2011; 1:51-58.
34. Kay CD, Hooper L, Kroon PA, Rimm EB, Cassidy A. Relative impact of flavonoid composition, dose and structure on vascular function: A systematic review of randomized controlled trials of flavonoid-rich food products. Mol. Nutr. Food Res. 2012; 256:1605-16.

35. Yang L, Jia Z, Yang L, et al. Exercise Protects against Chronic β-Adrenergic Remodeling of the Heart by Activation of Endothelial Nitric Oxide Synthase. PLoS One. 2014; 9:e96892.
36. Simko F, Bednarova KR, Krajcirovicova K, et al. Melatonin reduces cardiac remodeling and improves survival in rats with isoproterenol-induced heart failure. J. Pineal Res. 2014; 57:177-84.
37. Tsutsui H, Kinugawa S, Matsushima S. Oxidative stress and heart failure. Am. J. Physiol. Heart Circ. Physiol. 2011; 301:H2181-90.
38. Gaweł S, Wardas M, Niedworok E, Wardas P. Malondialdehyde (MDA) as a lipid peroxidation marker. Wiad. Lek. 2004; 57:453-5.
39. Rajadurai M, Prince PSM. Preventive effect of naringin on lipids, lipoproteins and lipid metabolic enzymes in isoproterenol-induced myocardial infarction in Wistar rats. J. Biochem. Mol. Toxicol. 2006; 20:191-197.
40. Murugesan M, Revathi R, Manju V. Cardioprotective effect of fenugreek on isoproterenol induced myocardial infarction in rats. Indian J Pharmacol. 2011; 43(5): 516–519.
41. Tang ELH, Rajarajeswaran J, Fung SY, Kanthimathi MS. Antioxidant activity of Coriandrum sativum and protection against DNA damage and cancer cell migration. BMC Complementary and Alternative Medicine. 2013; 13:347.
42. Giardano FJ. Oxygen, oxidative stress, hypoxia, and heart failure. J. Clin. Invest. 2005; 115:500-508.
43. Morimoto C, Kiyama A, Kameda K, Ninomiya H, Tsujita T, Okuda H. Mechanism of the stimulatory action of okadaic acid on lipolysis in rat fat cells. The Journal of Lipid Research. 2000; 41(2): 199–20.
44. Radhiga T, Rajamanickam C, Senthil S, Pugalendi KV. Effect of ursolic acid on cardiac marker enzymes, lipid profile and macroscopic enzyme mapping assay in isoproterenol-induced myocardial ischemic rats. Food Chem Toxicol. 2012; 50(11): 3971-7.
45. Dhanapakiam P, Joseph JM, Ramaswamy VK, Moorthi M, Kumar AS. The cholesterol lowering property of coriander seeds (Coriandrum sativum): mechanism of action. J Environ Biol. 2008; 29(1): 53-6.
46. Bafna A, Maheshwari RS, Ved RK, Sarkar PD, Batham AR. Study of atherogenic indices in nephrotic syndrome. Int. J. Biol. Med. Res. 2012; 3:2257-2260.
47. Ohkita M, Tawa M, Kitada K, Matsumura Y. Pathophysiological Roles of Endothelin Receptors in Cardiovascular Diseases. J. Pharmacol. Sci. 2012; 119:302 – 313.
48. Stenman E, Malmsjo M, Uddman E, Gido G, Wieloch T, Edvinsson L. Cerebral ischemia upregulates vascular endothelin ET(B) receptors in rat. Stroke. 2002; 33:2311-2316.
49. Zheng JP, Zhang Y, Edvinsson L, Hjalt T, Xu CB. NF-kappaB signaling mediates vascular smooth muscle endothelin type B receptor expression in resistance arteries. Eur. J. Pharmacol. 2010; 637:148–154.
50. Dimitrijevic I, Edvinsson ML, Chen Q, Malmsjo M, Kimblad PO, Edvinsson L. Increased expression of vascular endothelin type B and angiotensin type 1 receptors in patients with ischemic heart disease. BMC Cardiovasc. Disord. 2009; 9:40.

EFFECTS OF ISCHEMIC AND PROTON PUMP INHIBITORS PRECONDITIONING ON OXIDATIVE STRESS OF ISOLATED RAT HEART

Nevena Jeremic[1], Vladimir Zivkovic[2], Ivan Srejovic[2], Jovana Jeremic[3], Anica Petkovic[3], Jovana Bradic[3], Vladimir Jakovljevic[2, 4]
[1] Department of Pharmaceutical chemistry, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[2] Department of Physiology, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[3] Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[4]Department of Human Pathology, 1st Moscow State Medical University IM Sechenov, Moscow, Russia

EFEKTI ISHEMIJSKOG I PREKONDICIONIRANJA INHIBITORIMA PROTONSKE PUMPE NA OKSIDACIONI STRES IZOLOVANOG SRCA PACOVA

Nevena Jeremić[1], Vladimir Živković[2], Ivan Srejović[2], Jovana Jeremić[3], Anica Petković[3], Jovana Bradić[3], Vladimir Jakovljević[2, 4]
[1] Katedra za farmaceutsku hemiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[2] Katedra za fiziologiju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[3] Katedra za farmaciju, Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[4]Odsek za humanu patologiju, Prvi Moskovski državni medicinski univerzitet I.M. Sechenov, Moskva, Rusija

Received / Primljen: 11. 03. 2017. Accepted / Prihvaćen: 14. 03. 2017.

ABSTRACT

Aim of present study was to determine the participation of various biomarkers of oxidative damage: nitrite (NO_2^-), superoxide anion radicals (O_2^-), index of lipid peroxidation (TBARS) and hydrogen peroxide (H_2O_2) in coronary circulation after application of the different models of preconditioning such as ischemic and preconditioning with proton pump inhibitors.

Examining a biochemical markers of oxidative damage we did not notice any increased production values of any parameter, according to that we can hypothesize that possible occurrence of reperfusion injury after ischemia and PPIs preconditioning is not mediated by this mechanism.

Due to the very difficult and controversial application of ischemic preconditioning in clinical practice, the results of this study suggest that in the future proton pump inhibitors can contribute to the prevention of myocardial damage following ischemia

Keywords: *ischemic preconditioning, myocardial infarction, proton pump inhibitors, oxidative stress*

SAŽETAK

Cilj ove studije bio je ispitati učesće različitih biomarkera oksidacionog oštećenja : nitrita (NO_2^-), superoksid anjon radikala (O_2^-), lipidnih peroksida (TBARS) i vodonik peroksida (H_2O_2) pri primeni različitih vrsta prekondiconiranja kao sto su prekondicioniranje ishemijom i prekondicioniranje lekovima iz grupe inhibitora protonske pumpe.

S obzirom da ispitujući biohemijske markere oksidacionog oštećenja nismo uočili da je došlo do pojačane produkcije vrednosti bilo kog parametra, možemo pretpostaviti da eventualni nastanak reperfuzione povrede nakon prekondicioniranja ishemijom i lekovima iz grupe inhibitora protonske pumpe nije posredovan ovim mehanizmom.

Zbog veoma teške i kontroverzne primene ishemijskog prekondicioniranja u kliničkoj praksi, rezultati ove studije sugerišu da u budućnosti lekovi iz grupe inhibitora protonske pumpe mogu imati svoj doprinos u prevenciji oštećenja miokarda nakon ishemije.

Ključne reči: *ishemijsko prekondicioniranje, infarkt miokarda, inhibitori protonske pumpe, oksidacioni stres*

INTRODUCTION

There is no doubt that myocardial ischemia (MI) is one of the major causes of morbidity and mortality worldwide. Various medical and surgical strategies have been developed over the years in order to minimize the deep and devastating effects on metabolism and myocardial contractility as well as the sustainability of myocytes due to acute myocardial infarction (AMI). Approaches vary, but usually include the use of thrombolytic agents, antagonist β-adrenergic receptor, inhibitors of angiotensin converting enzyme (ACE), the use of antioxidants, the use of transluminal coronary angioplasty, coronary artery bypass grafting. One of the few interventions that are universally accepted and still causes a great deal of attention by the scientific world is certainly a phenomenon of heart ischemia preconditioning (1).

Over time, a growing number of studies observed different models and effects of preconditioning (2, 3). Preconditioning stimulus itself leads to the start of the whole cascade of endogenous adaptive mechanisms that ultimately result in the development of tolerance. It was also shown that preconditioning is powerful mechanism that protects the myocardium from ischemic damage, to reduce the occurrence of arrhythmia, and that has a role in maintaining contractility of the cardiac muscle (4).

UDK: 616.127; 616-008.9:[577.344:546.21
Ser J Exp Clin Res 2018; 19 (2): 131-139;
DOI: 10.1515/SJECR-2017-0008

Corresponding author: Vladimir Jakovljevic MD, PhD
Department of Physiology; Faculty of Medical Sciences; University of Kragujevac; Kragujevac, Serbia
Address: Svetozara Markovica 69; Phone number: +38134306800 ex 114; Email: drvladakgbg@yahoo.com

The mechanism of the formation of reactive oxygen species (reactive oxygen spieces - ROS) is very important in the process of reperfusion injury and experimental studies have shown that reperfusion after ischemia generates oxidative stress, which can then be intermediary for myocardial infarction. The oxygen that reaches infarction who had previously suffered ischemia, leads to the formation of free radicals, primarily superoxide, hydroxyl radicals, peroxynitrite, which just in the first few minutes of establishing blood flow leads to the development of reperfusion injury. ROS can occur as a result of the activation, and neutrophil accumulation during reperfusion resulting in the release of platelet activating factor derived from the endothelium, which attract neutrophils and enhance the production of ROS as well as the degree of reperfusion injury. During reperfusion, oxidative stress has also reduces the bioavailability of intracellular signaling molecules such as nitric oxide, thereby negating its cardio protection (5, 6).

Since the goal of any kind of preconditioning is to reduce infarct size, the available literature data that can be found today used a wide variety of pharmacological agents, among them also proton pump inhibitors (PPI).

Gomes and his associates in its two research looked at the role of omeprazole and pantoprazole as possible preconditioning agents. In a study with omeprazole is observed that the drug significantly contribute to a recovery in isolated rat heart after injury caused by myocardial ischemia and reperfusion. Similar conclusions are performed from a survey conducted with pantoprazole on isolated rat heart and same findings we have from our previous work (7-9).

According to all previous our aim was to determine the participation of various biomarkers of oxidative damage: nitrite (NO_2^-), superoxide anion radicals (O_2^-), index of lipid peroxidation (TBARS) and hydrogen peroxide (H_2O_2) in coronary circulation after application of the different models of preconditioning such as ischemic and preconditioning with proton pump inhibitors.

MATERIAL AND METHODS

Preparation of isolated rat hearts

The hearts of male Wistar albino rats (n=36, 12 in each experimental group, body mass 180–200 g) were excised and perfused on a Langendorff apparatus (Experimetria Ltd,1062 Budapest, Hungary). After a short-term ketamine/xylasin narcosis, animals were killed by cervical dislocation (Schedule 1 of the Animals/ Scientific Procedures, Act 1986, UK), and premedicated with heparin as an anticoagulant. After emergency thoracotomy and rapid cardiac arrest by superfusion with ice-cold isotonic saline, rapidly excised, the aortas were cannulated and retrogradely perfused under a constant perfusion pressure (CPP). The composition of the non-recirculating Krebs-Henseleit perfusate was as follows (mM): NaCl 118, KCI 4.7, $CaCI_2$ $x2H_2O$ 2.5, $MgSO_4$ $x7H_2O$ 1.7, $NaHCO_3$ 25, KH_2 PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95 % O_2 plus 5% CO_2 and warmed to 37°C (pH 7.4). Immediately after the restoration of normal heart rhythm, through the created entrance to the left atrium of the heart and damaged mitral valve, the sensor (transducer BS473-0184, Experimetria Ltd, Budapest, Hungary) was inserted into the left ventricle for continuous monitoring of cardiac function.

Physiological assay and experimental protocol

All study groups underwent 30 min perfusion at CPP of 70 cm H_2O. In control group (CG) after stabilization period, hearts were subjected to global ischemia (perfusion was totally stopped) for 20 minutes and 30 minutes of reperfusion (Protocol 1). Hearts of group II (IPC) were submitted to ischemic preconditioning lasting 2 minutes of ischemia and 4 minutes of reperfusion before global ischemia of 20 minutes and 30 minutes of reperfusion (Protocol 2). Third, fourth and fifth groups were groups with pharmacological preconditioning (proton pump inhibitors). All of these groups first underwent preconditioning lasting 5 minutes with 100μM of drug (omeprazole, pantoprazole and lansoprazole) then submitted 20 minutes of ischemia and 30 minutes of reperfusion (Protocol 3).

In control group, coronary venous effluent was collected in point of stabilization (S), in first point of reperfusion (R1) and on every 5 minutes in period of reperfusion (R1-R7). In group with ischemic preconditioning coronary venous effluent was collected in point of stabilization (S), in each point on every minute of 4 minute reperfusion period (RP1-RP4) and from first to the last point of 30 minutes reperfusion period on every 5 minutes (R1-R7). In groups of pharmacological preconditioning coronary venous effluent was collected in point of stabilization (S) and from first to the last point of 30 minutes reperfusion period on every 5 minutes (R1-R7).

Drugs

All drugs were purchased from Sigma–Aldrich Chemie GmbH, Germany.

Biochemical Assays

In the collected samples of coronary venous effluent, following markers of oxidative stress were measured spectrophotometrically: (1) index of lipid peroxidation (measured as TBARS—thiobarbituric acid reactive substances); (2) nitrites (NO_2^-); (3) hydrogen peroxide (H_2O_2); (4) superoxide anion radical (O_2^-).

Index of lipid peroxidation

The degree of lipid peroxidation in the coronary venous effluent was estimated by measuring TBARS using 1 % thiobarbituric acid (TBA) in 0.05 NaOH incubated with the coronary effluent at 100 °C for 15 min and read at 530 nm. Krebs–Henseleit solution was used as a blank probe (10).

Nitrite determination

NO rapidly decomposes to form stable metabolite nitrite/nitrate products. The nitrite (NO_2^-) level was measured as an index of NO production using the Griess reagent. A total of 0.5 ml of perfusate was precipitated with 200 µl of 30 % sulphosalicylic acid, vortexed for 30 min, and centrifuged at 3000×*g*. Equal volumes of the supernatant and Griess reagent, containing 1 % sulphanilamide in 5 % phosphoric acid/0.1 % naphthalene ethylenediamine dihydrochloride, were added, incubated for 10 min in the dark, and read at 543 nm. The nitrite levels were calculated using sodium nitrite as the standard (11).

Determination of superoxide anion radical

The level of the superoxide anion radical (O_2^-) was measured by nitro blue tetrazolium (NBT) reaction in Tris buffer with coronary venous effluent and read at 530 nm. Krebs–Henseleit solution was used as a blank probe (12).

Determination of hydrogen peroxide

The measurement of H_2O_2 is based on the oxidation of phenol red by H_2O_2 in a reaction catalyzed by horseradish peroxidase (HRPO) (13). A volume of 200 l of perfusate was precipitated with 800 l of fresh phenol red solution (PRS), along with 10 l of 1:20 HRPO (made ex tempore). An adequate volume of Krebs–Henseleit solution was used for a blank probe (instead of coronary venous effluent). The level of H_2O_2 was measured at 610 nm.

Statistical analysis

For statistical analysis we examined three measured points, first point was stabilization (S), second was the first and the last point of 30 minutes reperfusion period (R1 and R7). Values are expressed as mean ± SE. Statistical analysis was performed by ANOVA test. P values lower than 0.05 were considered to be significant.

Ethical Approval

The experimental protocol was approved by the Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac, number 01-12149 and by Ministry of Agriculture, Forestry and Water Management, Authority for Veterinary of Serbia number 323-07-09426/2013-05.

RESULTS

Effects of ischemic and PPI preconditioning on index of lipid peroxidation

Control group and group with pantoprazole leaded to statistically significant difference between first and last point of reperfusion. Although in all other groups we noticed decreased levels of TBARS in reperfusion period that results were not statistically significant. Except in group were we applied lansoprazole, there were no difference between values in point of stabilization compared to last point of reperfusion (Table 1 and Figure 1).

Effects of ischemic and PPI preconditioning on nitrites

In all experimental groups we found decreased levels of nitrites in reperfusion period. However in control and group with lansoprazole there were no difference among values in first and last point of reperfusion. When we compared values in S point with values in R7 point we notice that there were no statistical difference between these points in control group and in groups with ischemic and lansoprazole preconditioning (Table 2, Figure 2).

Table 1. Statistical significance and results for all observed groups

Index of lipid peroxidation (TBARS)					
Points of interest/ Groups	**Control**	**IP**	**OP**	**LP**	**PP**
S *vs* R1	***P<0.05****	P>0.05	P>0.05	P>0.05	P>0.05
S *vs* R7	***P<0.05****	P>0.05	***P<0.05****	***P<0.01****	P>0.05
R1 *vs* R7	***P<0.01****	P>0.05	P>0.05	P>0.05	***P<0.05****
Decrease (-) or increase (+) values R7 *vs* R1 (%)	*-43,92*	*-37,26*	*- 58,98*	*-23,28*	*-27,83*
Decrease (-) or increase (+) values R7 *vs* S (%)	*-25,16*	*-15,80*	*-55,66*	*-25,17*	*-19,81*

Table 2. Statistical significance and results for all observed groups

Nitrites (NO_2^-)					
Points of interest/ Groups	**Control**	**IP**	**OP**	**LP**	**PP**
S *vs* R1	P>0.05	***P<0.01****	P>0.05	***P<0.05****	***P<0.01****
S *vs* R7	P>0.05	P>0.05	***P<0.01****	***P<0.05****	***P<0.05****
R1 *vs* R7	P>0.05	***P<0.01****	***P<0.01****	P>0.05	***P<0.01****
Decrease (-) or increase (+) values R7 *vs* R1 (%)	*-39,79*	*-33,69*	*- 46,97*	*-29,86*	*-28,83*
Decrease (-) or increase (+) values R7 *vs* S (%)	*-33,18*	*-4,99*	*-34,16*	*-15,97*	*-14,79*

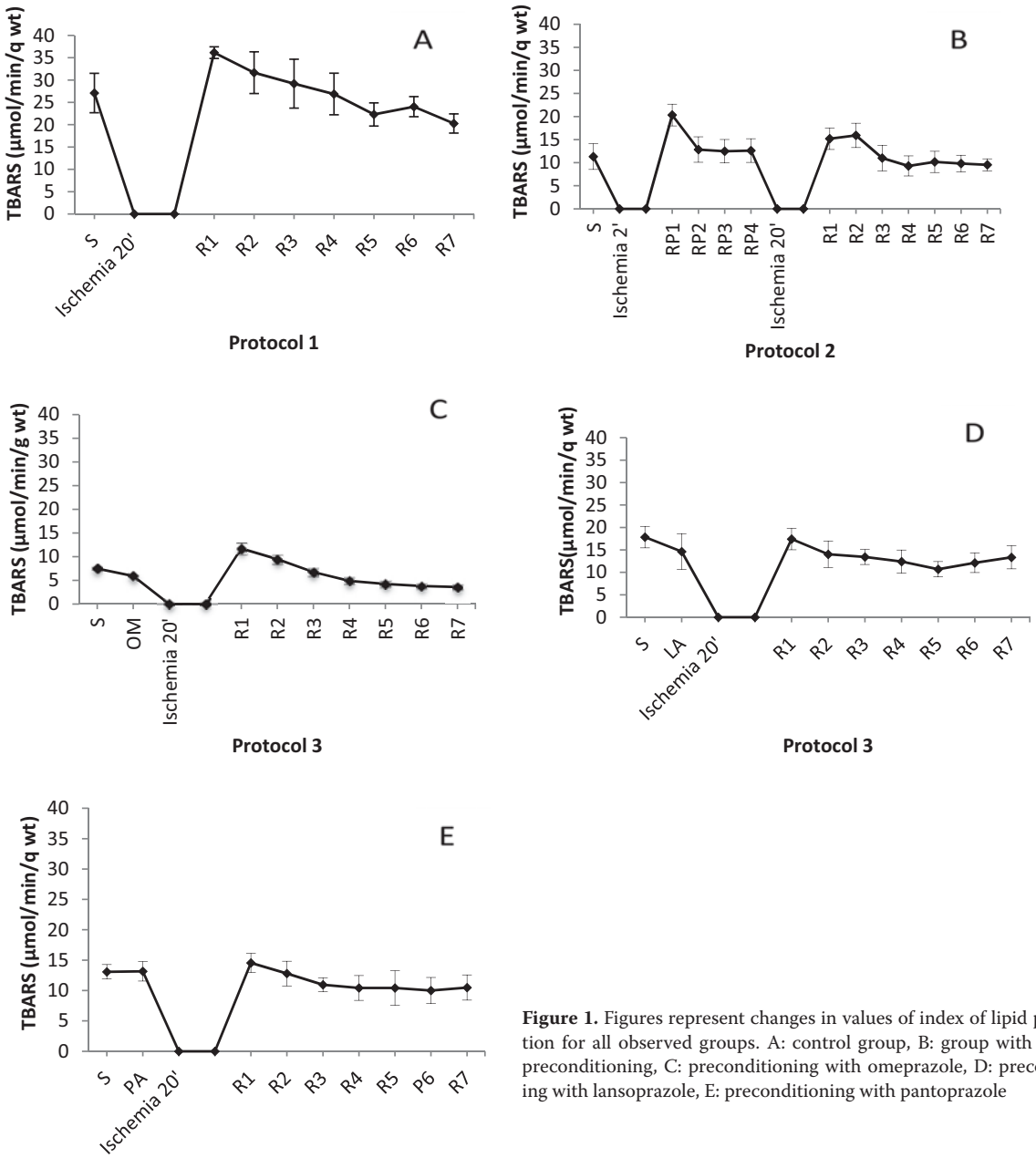


Figure 1. Figures represent changes in values of index of lipid peroxidation for all observed groups. A: control group, B: group with ischemic preconditioning, C: preconditioning with omeprazole, D: preconditioning with lansoprazole, E: preconditioning with pantoprazole

Effects of ischemic and PPI preconditioning on superoxide anion radical

In groups with ischemic and preconditioning with pantoprazole we found statistically significant increase in values of superoxide anion radical in period of reperfusion. Moreover in group with lansoprazole preconditioning in same examined period we showed statistically significant decrease of these values. There were no significant difference between values in S point and values in R7 point in examined groups (Table 3, Figure 3).

Effects of ischemic and PPI preconditioning on hydrogen peroxide

Ischemic and PPI preconditioning had statistically significant effect on values of hydrogen peroxide in reperfusion period. In these groups we found decreased values in R7 point compared to R1 point. There were no significant changes in control group. Moreover, in control group, group with ischemic and omeprazole preconditioning we found that there is no difference between values in S point compared to values in R7 point of examined parameter (Table 4, Figure 4).

DISCUSSION

Determining the level molecule such as: nitric oxide in the form of nitrite, superoxide anion radicals and hydrogen peroxide and marker of oxidative damage (index of lipid peroxidation) in the coronary venous effluent, we wanted to determine whether ischemic preconditioning and phar-

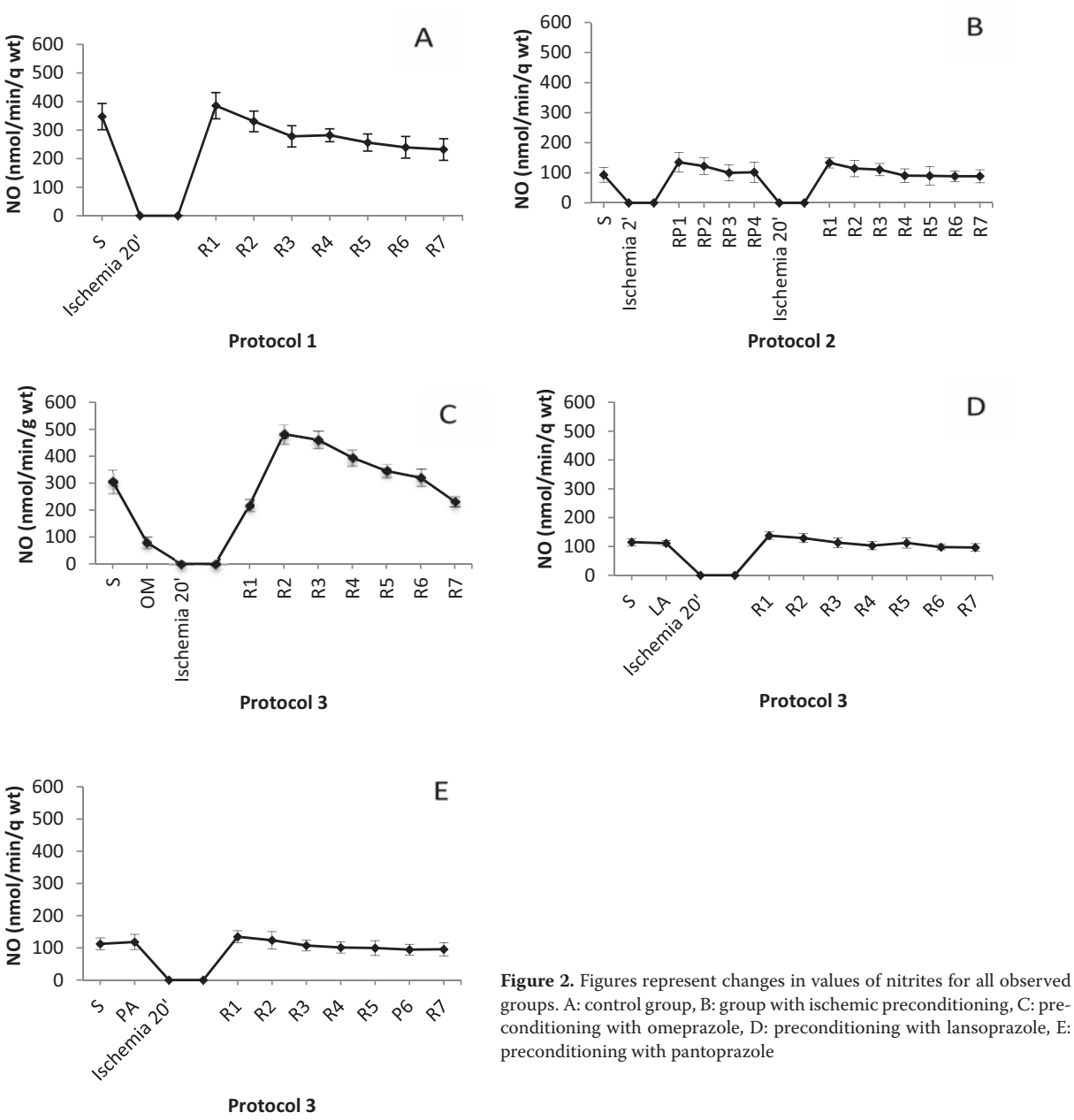


Figure 2. Figures represent changes in values of nitrites for all observed groups. A: control group, B: group with ischemic preconditioning, C: preconditioning with omeprazole, D: preconditioning with lansoprazole, E: preconditioning with pantoprazole

macological preconditioning with PPIs have a positive or negative effect on the free radical production during reperfusion. Myocardial reperfusion injury is an essential consequence of re-establishing the flow after ischemia. Although the exact mechanisms that can protect the myocardium from the occurrence of the injury or which it can alleviate not yet been adequately investigated, the interest in this problem still remain.

Our results show that drugs called proton pump inhibitors significantly reduced the concentration of nitric oxide in reperfusion period compared to the last point and the point of stabilization, but that is largely decreased of nitrite caused by omeprazole. Control group had decrease in nitrite values for 33.18% in reperfusion period. The less reduction in the value of exempt nitrite showed the group where myocardium was preconditioned with ischemia (Table 2, Figure 2).

The importance of nitric oxide in the I/R injury of the myocardium is especially great due to its reaction with superoxide anion radical resulting in the generation of peroxynitrite (14-16). Peroxynitrite ($ONOO^-$) can cause oxidation of the protein as well as lipid peroxidation (14). Our results could be interpreted in the light of these facts, because all groups reduced the release of superoxide anion radicals which can be spent on administration to create peroxynitrite by reaction with nitrogen oxide whose values are also in decline.

Review that at the end of the twentieth century posted Nonami et al., indicates that based on a critical review of previously published works, one cannot claim with cer-

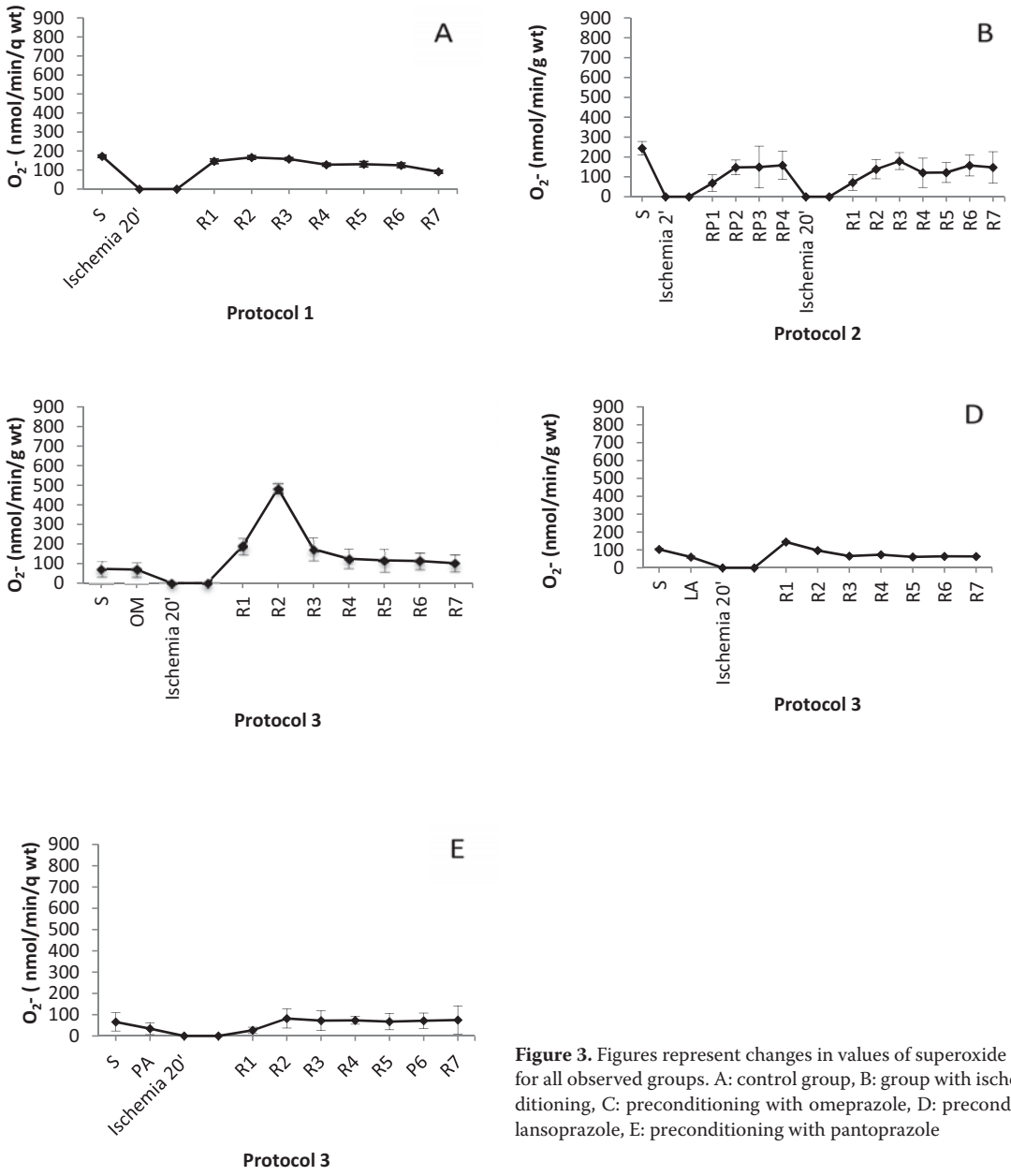


Figure 3. Figures represent changes in values of superoxide anion radical for all observed groups. A: control group, B: group with ischemic preconditioning, C: preconditioning with omeprazole, D: preconditioning with lansoprazole, E: preconditioning with pantoprazole

Table 3. Statistical significance and results for all observed groups

Superoxide anion radical (O_2^-)					
Points of interest/ Groups	**Control**	**IP**	**OP**	**LP**	**PP**
S *vs* R1	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
S *vs* R7	P>0.05	P>0.05	P>0.05	P>0.05	P>0.05
R1 *vs* R7	P>0.05	***P<0.05****	P>0.05	***P<0.05****	***P<0.05****
Decrease (-) or increase (+) values R7 *vs* R1 (%)	*-37,72*	*+107,28*	*- 37,73*	*-56,22*	*+181,83*
Decrease (-) or increase (+) values R7 *vs* S (%)	*-47,30*	*-39,46*	*-26,92*	*-38,57*	*-13,87*

tainty that neither donors nor precursors of nitric oxide, as well as inhibitors of nitric oxide synthase sufficient to prevent I/R injuries. In conclusion they indicated that nitric oxide may have different effects in terms of myocardial protection and all depending on the time of its application (17).

Although the superoxide anion radical is a highly reactive molecule, in the physiological conditions this molecule almost immediately after the creation has been translated into hydrogen peroxide using mitochondrial (Mn-SOD) and cytosolic (Cu / Zn-SOD), superoxide dismutase. Further, hydrogen peroxide under the action of the enzyme catalase is converted into oxygen and water. However, under certain circumstances, superoxide anion radical can avoid the cascading time, with consequent production of ROS leading to oxidative damage (14, 18, 19).

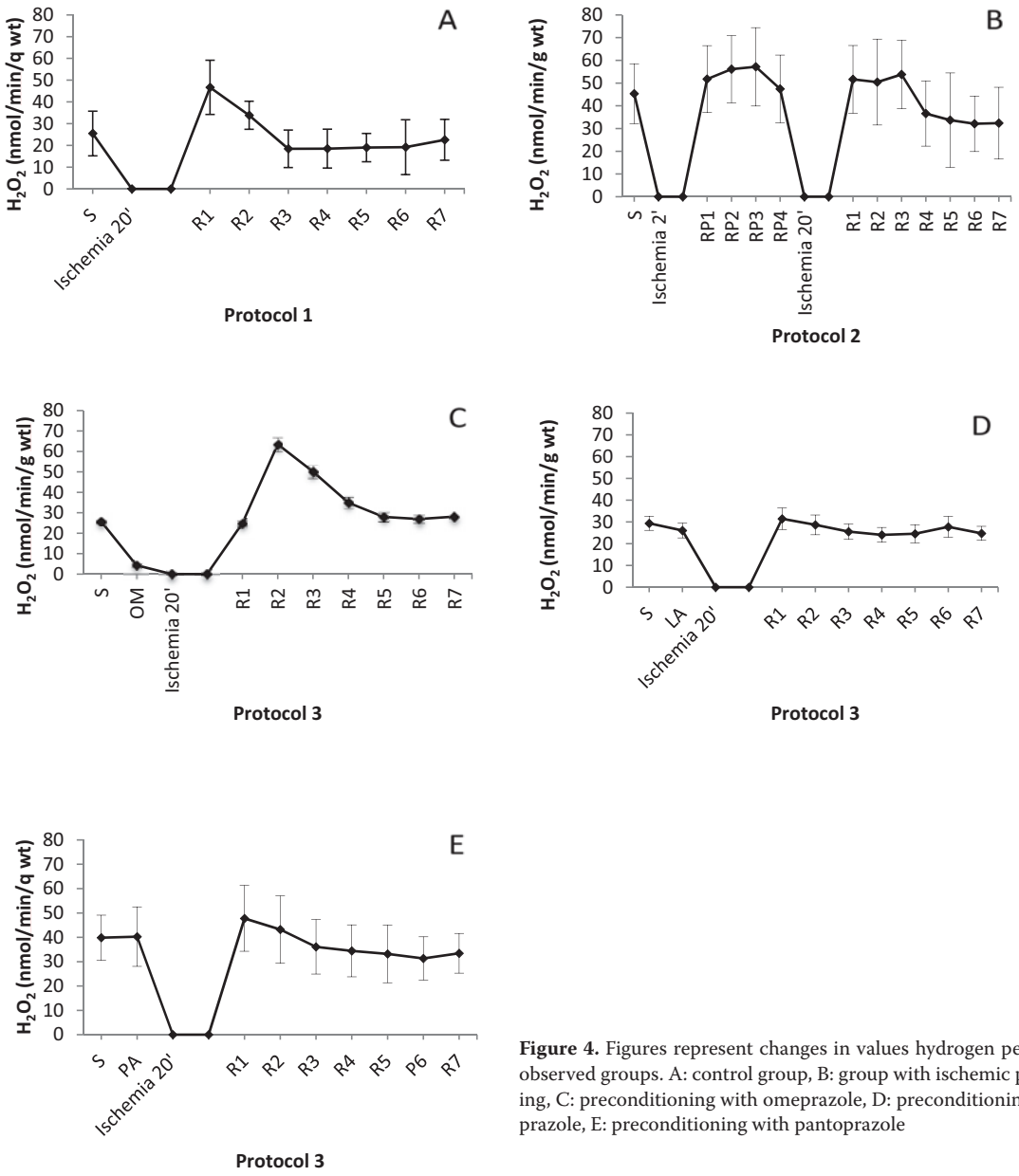


Figure 4. Figures represent changes in values hydrogen peroxide for all observed groups. A: control group, B: group with ischemic preconditioning, C: preconditioning with omeprazole, D: preconditioning with lansoprazole, E: preconditioning with pantoprazole

Table 4. Statistical significance and results for all observed groups

Hydrogen-peroxide (H_2O_2)					
Points of interest/ Groups	**Control**	**IP**	**OP**	**LP**	**PP**
S *vs* R1	***P<0.05****	P>0.05	P>0.05	***P<0.05****	***P<0.05****
S *vs* R7	P>0.05	P>0.05	P>0.05	***P<0.01****	***P<0.05****
R1 *vs* R7	P>0.05	***P<0.05****	***P<0.01****	***P<0.05****	***P<0.01****
Decrease (-) or increase (+) values R7 *vs* R1 (%)	***-51,65***	***-37,29***	***- 22,57***	***-21,27***	***-30,02***
Decrease (-) or increase (+) values R7 *vs* S (%)	***-11,40***	***-28,56***	***-26,85***	***-15,70***	***-16,10***

Our results may be seen from the table and graphics (Table 3, Chart 3), indicating reduced release of superoxide anion radical, which is not statistically significant in either study group when comparing the last point of reperfusion and values at point of stabilization. Decrease in superoxide anion radicals by active metabolite of omeprazole showed Wandall early nineties of the last century on the model of isolated polymorphous (20). In a study in isolated rabbit hearts, Omar et al. came to the conclusion that ischemic preconditioning is not mediated by superoxide anion radicals. Research by various laboratories indicated that the generation of ROS plays a vital role in the oxidative damage after ischemia and in the protection of the myocardium. This paradox may be explained by the specific regulatory resources ROS (14).

When it comes to hydrogen peroxide, we saw that after the application of drugs from the group of PPIs percentage drop in values when we compared the point R7 relative to S.

The values of hydrogen peroxide from the coronary venous effluent had the same trend and in the control group or the groups with the ischemic preconditioning (Table 4, Chart 4).

Pantoprazole in a study conducted in an animal model of rats showing its anti-oxidant and cytoprotective effects (21, 22). Same ability lansoprazole showed also (23). As far as preconditioning with ischemia, Gozal et al. on the model of the dog heart have shown that this type of prevention of myocardium injury leads to a reduction in the concentration of hydroxyl radicals, and thus concluded that ischemic preconditioning does not cause oxidative damage (24).

Although in our study there was no evidence that preconditioning ischemia as well as proton pump inhibitors reduces the value of hydroxyl radicals, due to the overall reduction of all parameters of oxidative damage it can be indicated possible the same scenarios as in the previously described studies.

The process of lipid peroxidation may occur through non-enzymatic and be mediated by reactive oxygen species resulting in the formation of malondialdehyde (MDA). Exposure to high levels of lipid peroxidation products can cause a variety of cellular responses, ranging from acute toxic to the inhibition of cell proliferation (25).

Our results showed that in all groups, there was a decrease in the values of superoxide anion radicals and hydrogen peroxide. How O_2^- leads to lipid peroxidation of membranes of endothelial cells, reduction of the levels of these pro-oxidant is in correlation with the lower values of lipid peroxidation (TBARS) (16). Reducing TBARS values can be traced through all the experimental groups, which clearly shows the percentage drop in the value of the point R7 in relation to point of stabilization. The difference between the values at the end of reperfusion, and the basal value at the beginning of the experiment did not differ statistically in a group where we executed ischemic preconditioning and preconditioning with pantoprazole. This might be a sign that in these groups after a large percentage drop during reperfusion period at the end of reperfusion established that lipid peroxidation and there were no deferens between R7 and S (Table 1, Figure 1).

In the group where omeprazole was applied we found that there has been a decline in the values in the reperfusion period compared to stabilization for 55.56%. Moreover as in this group came up almost the largest decrease in the concentration of hydrogen peroxide, it can be assumed that lipid peroxidation due to at least because the minimum production of hydroxyl radicals. Hayashi et al. on the model of isolated rat liver showed that omeprazole decreases the values of lipid peroxidation (26) while on indomethacin-induced gastritis in rats demonstrated that administration of omeprazole prevent an increase in TBARS values (27).

CONCLUSION

Examining a biochemical markers of oxidative damage we did not notice any increased production values of any parameter, according to that we can hypothesize that possible occurrence of reperfusion injury after ischemia and PPIs preconditioning is not mediated by this mechanism.

Due to the very difficult and controversial application of ischemic preconditioning in clinical practice, the results of this study suggest that in the future drugs called proton pump inhibitors can contribute to the prevention of myocardial damage following ischemia, especially because these days this group of drugs is one of the most prescribing.

Acknowledgment

This study is supported by Junior Project (JP 3/11) of Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia.

REFERENCES

1. Przyklenk, K., & Kloner, R. A. (1998). Ischemic preconditioning: exploring the paradox. Prog Cardiovasc Dis, 40(6), 517-547.
2. Ara, J., Fekete, S., Frank, M., Golden, J. A., Pleasure, D., & Valencia, I. (2011). Hypoxic-preconditioning induces neuroprotection against hypoxia-ischemia in newborn piglet brain. Neurobiol Dis, 43(2), 473-485.
3. Cantagrel, S., Krier, C., Ducrocq, S., Bodard, S., Payen, V., Laugier, J., Chalon, S. (2003). Hypoxic preconditioning reduces apoptosis in a rat model of immature brain hypoxia-ischaemia. Neurosci Lett, 347(2), 106-110.
4. Altug, S., Demiryurek, A. T., Kane, K. A., & Kanzik, I. (2000). Evidence for the involvement of peroxynitrite in ischaemic preconditioning in rat isolated hearts. Br J Pharmacol, 130(1), 125-131.
5. Verma, S., Maitland, A., Weisel, R. D., Li, S. H., Fedak, P. W., Pomroy, N. C., Rao, V. (2002). Hyperglycemia exaggerates ischemia-reperfusion-induced cardiomyocyte injury: reversal with endothelin antagonism. J Thorac Cardiovasc Surg, 123(6), 1120-1124.
6. Yellon, D. M., & Hausenloy, D. J. (2007). Myocardial reperfusion injury. N Engl J Med, 357(11), 1121-1135.
7. Gomes, O. M., Magalhaes Mde, M., & Abrantes, R. D. (2010). Myocardium functional recovery protection by omeprazole after ischemia-reperfusion in isolated rat hearts. Rev Bras Cir Cardiovasc, 25(3), 388-392.
8. Gomes, O. M., Magalhaes Mde, M., Abrantes, R. D., & Kallas, E. (2011). Pantoprazole provides myocardial protection similar to ischemic preconditioning: experimental study of isolated hearts of rats. Rev Bras Cir Cardiovasc, 26(3), 433-439.
9. Jeremic, N., Petkovic, A., Srejovic, I., Zivkovic, V., Djuric, D., & Jakovljevic, V. (2015). Effects of ischemia and omeprazole preconditioning on functional recovery of isolated rat heart. Rev Bras Cir Cardiovasc, 30(2), 266-275.
10. Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem, 95(2), 351-358.

11. Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., & Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem, 126(1), 131-138.
12. C Auclair, E Voisin. (1985). Nitroblue tetrazolium reduction Vol. 123. Handbook of methods for oxygen radical research (pp. 123–132).
13. Pick, E., & Keisari, Y. (1980). A simple colorimetric method for the measurement of hydrogen peroxide produced by cells in culture. J Immunol Methods, 38(1-2), 161-170.
14. Goswami, S. K., Maulik, N., & Das, D. K. (2007). Ischemia-reperfusion and cardioprotection: a delicate balance between reactive oxygen species generation and redox homeostasis. Ann Med, 39(4), 275-289.
15. Kurzelewski, M., Czarnowska, E., & Beresewicz, A. (2005). Superoxide- and nitric oxide-derived species mediate endothelial dysfunction, endothelial glycocalyx disruption, and enhanced neutrophil adhesion in the post-ischemic guinea-pig heart. J Physiol Pharmacol, 56(2), 163-178.
16. Raedschelders, K., Ansley, D. M., & Chen, D. D. (2012). The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. Pharmacol Ther, 133(2), 230-255.
17. Nonami, Y. (1997). The role of nitric oxide in cardiac ischemia-reperfusion injury. Jpn Circ J, 61(2), 119-132.
18. Hess, M. L., & Manson, N. H. (1984). Molecular oxygen: friend and foe. The role of the oxygen free radical system in the calcium paradox, the oxygen paradox and ischemia/reperfusion injury. J Mol Cell Cardiol, 16(11), 969-985.
19. Pagliaro, P., & Penna, C. (2015). Redox signalling and cardioprotection: translatability and mechanism. Br J Pharmacol, 172(8), 1974-1995.
20. Wandall, J. H. (1992). Effects of omeprazole on neutrophil chemotaxis, super oxide production, degranulation, and translocation of cytochrome b-245. Gut, 33(5), 617-621.
21. Hackert, T., Tudor, S., Felix, K., Dovshanskiy, D., Hartwig, W., Simon, W. A., & Werner, J. (2010). Effects of pantoprazole in experimental acute pancreatitis. Life Sci, 87(17-18), 551-557.
22. Simon, W. A., Sturm, E., Hartmann, H. J., & Weser, U. (2006). Hydroxyl radical scavenging reactivity of proton pump inhibitors. Biochem Pharmacol, 71(9), 1337-1341.
23. Biswas, K., Bandyopadhyay, U., Chattopadhyay, I., Varadaraj, A., Ali, E., & Banerjee, R. K. (2003). A novel antioxidant and antiapoptotic role of omeprazole to block gastric ulcer through scavenging of hydroxyl radical. J Biol Chem, 278(13), 10993-11001.
24. Gozal, Y., Chevion, M., Elami, A., Berenshtein, E., Kitrossky, N., & Drenger, B. (2005). Ischaemic preconditioning but not isoflurane prevents post-ischaemic production of hydroxyl radicals in a canine model of ischaemia-reperfusion. Eur J Anaesthesiol, 22(1), 49-55.
25. Doucet, A. (1997). H+, K(+)-ATPASE in the kidney: localization and function in the nephron. Exp Nephrol, 5(4), 271-276.
26. Hayashi, H., Shimamoto, K., Taniai, E., Ishii, Y., Morita, R., Suzuki, K., Mitsumori, K. (2012). Liver tumor promoting effect of omeprazole in rats and its possible mechanism of action. J Toxicol Sci, 37(3), 491-501.
27. Petronilho, F., Araujo, J. H., Steckert, A. V., Rezin, G. T., Ferreira, G. K., Roesler, R., Streck, E. L. (2009). Effect of a gastrin-releasing peptide receptor antagonist and a proton pump inhibitor association in an animal model of gastritis. Peptides, 30(8), 1460-1465.

UBIQUINONE PLASMA LEVELS ARE CORRELATED WITH BRAIN NATRIURETIC PEPTIDE PLASMA LEVELS IN PATIENTS WITH CHRONIC HEART FAILURE: THE POTENTIAL OF COENZYME Q10 COMBINED THERAPY

Sizova Zh.M.[1], Zakharova V.L.[1], Alibeyli K.A.[1], Medvedev O.S.[2], Shikh E.V.[1], Bolevich S.B.[1], Bolevich S.S.[1], Vorobyev S.I.[1], Votrin S.V.[1], Padalko V.V.[1], Novikov A.A.[3],Omarov I.A.[4]

[1]I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia

[2]Lomonosov Moscow State University, Moscow, Russia

[3]The Federal state budgetary institution "The Federal bureau for medical-social expertise" of Ministry of labour and social protection of the Russian Federation (FSBI FB MSE of Mintrud of Russia), Moscow, Russia

[4]RUDN University, Moscow, Russia

POVEZANOST PLAZMA NIVOA UBIHINONA SA NIVOEM MOŽDANOG NATRIURETSKOG PEPTIDA KOD PACIJENATA SA HRONIČNOM BOLEŠĆU SRCA: EFEKTI KOMBINOVANE TERAPIJE KOENZIMOM Q10

Sizova Zh.M.[1], Zakharova V.L.[1], Alibeyli K.A.[1], Medvedev O.S.[2], Shikh E.V.[1], Bolevich S.B.[1], Bolevich S.S.[1], Vorobyev S.I.[1], Votrin S.V.[1], Padalko V.V.[1], Novikov A.A.[3],Omarov I.A.[4]

[1]Prvi moskovski državni univerzitet Sechenov, Moskva, Rusija

[2]Lomonosov državni univerzitet, Moskva, Rusija

[3]Federalna državna budžetska institucija "Savezni biro za medicinsko-socijalnu ekspertizu", Moskva, Rusija Ministarstva rada i socijalne zaštite Ruske Federacije (FSBI FB MSE Rusije), Moskva, Rusija

[4]Ruski univerzitet prijateljstva naroda, Moskva, Rusija

Received / Primljen: 22.04.2018. Accepted / Prihvaćen: 24.04.2018

ABSTRACT

Despite the association of a worse HF-related clinical status with lower CoQ10 levels, the prognostic use of CoQ10 is controversial. The aim of this study is to optimize pharmacotherapy for patients with ischaemic CHF, based on the clinical and functional parameters of the heart and brain natriuretic peptide (BNP) plasma levels, which are correlated with the CoQ10 plasma levels, and to assess patient prognosis after receiving CoQ10 therapy. This prospective clinical study included 75 patients aged 56 to 63 years old with coronary heart disease (CHD) classified as class I–III according to the NYHA classification. After assessment of the clinical-instrumental characteristics of the CVD course (complaints, medical history, physical examination, a 6-minute walk test, echocardiography, and test for reactive hyperaemia), we determined the BNP level and CoQ10 plasma levels. At the same time, we assessed the efficacy of CoQ10 treatment (at a dose of 60 mg/per day) and tolerability in CVD-combined therapy during a follow-up of 12 weeks. CoQ10 supplementation in HF patients induced improvements in their functional cardiac parameters, such as the ejection fraction. Our results suggest that supplemental CoQ10 may be a useful option for effective management of heart failure and warrant future adequately powered randomized controlled trials of CoQ10 supplementation in patients with HF.

Key words: *coenzyme Q10-combined therapy, brain natriuretic peptide, chronic heart failure*

SAŽETAK

Uprkos povezanosti lošeg HF kliničkog statusa sa nižim nivoima CoQ10, prognostička upotreba CoQ10 je kontroverzna. Cilj ove studije je razviti optimalnu farmakoterapiju za pacijente sa ishemijskom bolešću srca na osnovu kliničkih i funkcionalnih parametara srca i plazma nivoa natriureznog peptida B, koji je koreliran sa plazma nivoom CoQ10, kao i da se proceni ishod terpije CoQ10 (BNP). Ova prospektivna klinička studija uključila je 75 pacijenata starosti od 56 do 63 godina zivota sa koronarnom bolešću srca (CHD) klasifikovanim kao NIHA klasa I-III. Nakon procene kliničkih karakteristika CVD statusa (simptomi, medicinska istorija, fizički pregled, 6-minutni test hoda, ehokardiografija, test sa reaktivnom hiperemijom), merili smo nivoe BNP i CoQ10 u plazmi. Istovremeno, procenili smo efikasnost terapije CoQ10 (u dozi od 60 mg/dnevno) i toleranciju u kombinovanoj CVD terapiji tokom 12 nedelja. Suplementacija CoQ10 kod pacijenata sa HF je dovela do poboljšanja funkcionalnih parametara srca, kao što je ejekciona frakcija. Naši rezultati sugerišu da suplementacija CoQ10 može biti korisna opcija za efikasno upravljanje srčanim popuštanjem i obezbeđuju osnovu za buduće randomizirane i kontrolisane studije koje bi ispitivale suplementaciju CoQ10 kod pacijenata sa HF.

Kljucne reci: *koenzim Q10 kombinovana terapija, mozdani nstriurezni peptid, hronična bolest srca*

UDK: 615.356:577.161.6; 616.12-036.1-085.356
Ser J Exp Clin Res 2018; 19 (2): 141-149;
DOI: 10.2478/SJECR-2018-0012

Corresponding author: Prof. Sergey Bolevich, MD, PhD
Department of Human Pathology, First Moscow State Medical, University IM Sechenov, Trubetskaya street 8, str. 2 119991, Moscow, Russia,
e-mail: bolevich2011@yandex.ru

INTRODUCTION

In recent decades, the dominance of cardiovascular diseases (CVD), as major contributors to the total mortality of the employable population, has emerged. Chronic heart failure (CHF) plays a special role in the death rate because of its unfavourable course and prognosis. The results of the Framingham Heart Study indicated that CHF patients make up 2.5 percent of all Americans above 45 years of age or 5 million cases in total (550,000 new cases of CHF per year) (1, 2).

The current understanding of CHF pathophysiology is best described by the neurohormonal hypothesis. The use of renin-angiotensin-aldosterone system inhibitors and beta-blockers in clinical practice has led to a reliable reduction of total and cardiovascular mortality in patients with CHF (3, 4). However, apart from neurohormonal activation, increased production of pro-inflammatory cytokines following the immunoinflammatory response and initiation of lipid peroxidation play important roles in the pathophysiology of CVD. Increased reactive oxygen species (ROS) production leads to endothelial injury and reduction of nitric oxide production, triggers apoptosis, and has a negative inotropic effect (5, 6).

A potential treatment that option focuses on reducing symptoms and improving quality of life and prognosis of patients with CVD is the correction of myocardial oxidative injury by means of CoQ10 therapy (7). Coenzyme Q10 (CoQ10) is an endogenously synthesised and diet-supplied lipid-soluble cofactor that functions in the mitochondrial inner membrane to transfer electrons from complexes I and II to complex III (6, 7). Coenzyme Q10 (CoQ10), which is present in all cells of the human body, is essential for adenosine triphosphate (ATP) synthesis (5, 8). The reference range of reduced CoQ10 for children (younger than 18 years of age) is 320–1376 μg/L and for adults (aged 18 years or older) is 415–1480 μg/L. The reference values for total CoQ10 for children and adults are 320–1558 μg/L and 433–1532 μg/L, respectively. The normal percentage of reduced CoQ10 in children varies from 93% to 100%, while in adults, it falls between 92% and 98% (8).

Under experimental conditions, it was also shown that apart from increased reactive oxygen species production, CVD is accompanied by suppression of myocardial antioxidant systems (9). In the case of high levels of oxidative stress, CoQ10 reduces the nitric oxide inactivation rate (wherein NO is converted into peroxynitrite), thus protecting the vascular wall (10). It is well known that tissues with high energy requirements or metabolic activity, such as the heart, liver, kidneys, and muscles, contain the highest concentrations of CoQ10 (6, 9). Cardiomyocytes contain the highest concentration of ubiquinone due to their high energy requirements. It was established that plasma CoQ10 is progressively reduced with the increasing severity of CVD (11, 12).

Biomarker identification is a possible approach to estimating the severity of CVD and assessing its prognosis. In this procedure, determination of the B-type of brain natriuretic peptide plasma levels is conducted (13, 14). Increased levels of BNP are identified as an independent assessment criterion of CVD severity and a mortality predictor of CVD (13, 14).

Preclinical data have provided information across a variety of models that support the pathophysiological role of CoQ10 depletion in HF and other cardiovascular diseases as well as the concept of improved outcomes with CoQ10 supplementation (15). A meta-analysis of 13 randomized, controlled, blind trials indicated that CoQ10-combined therapy significantly improved the New York Heart Association (NYHA) functional class, increased physical activity tolerance, and reduced the number of hospitalizations due to CHF decompensation (16, 17). A number of studies indicate that application of CoQ10 at a daily dose of 100 – 200 mg results in increased contractility of the heart muscle (18). However, the results of some studies have been equivocal (18, 19). The possible reason for the limited efficacy of CoQ10-combined therapy may be the use of low doses.

There have been a large number of trials examining the effect of CoQ10 in HF conducted over the past 30 years. Despite the association of a worse HF-related clinical status with lower CoQ10 levels, the prognostic use of CoQ10 is controversial. The aim of this study is to develop pharmacotherapy optimization for patients with ischaemic CHF based on the clinical and functional parameters of the heart and brain natriuretic peptide (BNP) plasma levels, which are correlated with the CoQ10 plasma levels, and to assess patient prognosis after CoQ10 therapy.

PATIENTS AND METHODS

Ethical Approval

All patients before inclusion in the study provided informed consent. The study was approved by the Committee of Ethics of the local institution (IM Sechenov First Moscow State Medical University, Moscow, Russia) and conducted according to the principles of the Declaration of Helsinki.

Patients and study design

This prospective clinical study included 75 patients aged 56 to 63 years old with (CHD) classified as class I–III according to the NYHA classification. The inclusion criteria were the presence of CHF symptoms, left ventricular ejection fraction under 45% as confirmed by echocardiography and a previous myocardial infarction. Patients who were on standard therapy (beta-blockers, angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists, diuretics, statin nitrates and antiplatelet therapy) were also included.

After assessment of the clinical-instrumental characteristics of the CVD course (complaints, medical history, physical examination, a 6-minute walk test, echocardiog-

raphy, and a test for reactive hyperaemia), we determined the levels of BNP and plasma CoQ10. At the same time, we assessed the efficacy of the CoQ10 treatment (at a dose of 60 mg/per day) and its tolerability in CVD-combined therapy during a follow-up of 12 weeks. The efficacy and tolerability of the CoQ10-combined therapy was assessed according to the clinical manifestation dynamics of CHF, CHF functional class (NYHA), exercise tolerance, morphological and functional cardiac parameters and changes in plasma concentrations of NT-proBNP and CoQ10. All of the abovementioned parameters were assessed two times, at the beginning of the study (baseline) and after 12 weeks of follow-up.

Assessment of the patient clinical status

Assessment of the patient clinical status was based on analyses of complaints, a physical examination, the results of a 6-minute walk test, and morphological and functional parameters of the heart on echocardiography.

To assess endothelial-dependent vasodilatation, a Doppler evaluation of changes in the brachial artery diameter during reactive hyperaemia (HR) was used. The results were considered satisfactory if the brachial artery diameter, following the release of reactive hyperaemia, increased by more than 10%. In the case of a negative test result, endothelial dysfunction was diagnosed.

Biochemical analyses

Determination of N-terminal pro B-type natriuretic peptide (NT-proBNP)

The determination of the N-terminal pro B-type natriuretic peptide (NT-proBNP) plasma level was carried out using an automated immunochemiluminescence assay based on the sandwich principle ("Biomedica NT-proBNP") (20).

Determination of Coenzyme Q10 plasma levels

The determination of the Coenzyme Q10 plasma level was carried out using an isocratic method of reverse-phase high-performance liquid chromatography (HPLC). We used the Stayer liquid chromatograph system, Akvilon, Russia, Phenomenex Luna, with a 5 μm, 4.6×150 mm C18 column and ESA Coulochem II 5010 Electrochemical Detector. Chromatogram registration and processing were performed using the Environmental Sciences Associate, Inc., USA software (21).

Statistical analyses

Statistical analyses were carried out with the software package SPSS 11.5 for Windows. Nonparametric methods were used in the case of a non-Gaussian distribution of data, andalong with Spearman correlation analysis, the Wilcoxon test for paired comparisons, and the median test (a special case of the chi-square test used for multiple independent samples). The linear relationship intensity between independent and dependent variables, accounted for the influence of other variables and, was determined via multiple linear regression analysis. Data are presented as the median (Me) and range of the upper and lower quartiles based on a non-parametric distribution of data. Differences were considered significant at $p<0.05$.

RESULTS

Socio-demographic and clinical characteristics of the study group

This study consisted of 75 CHF patients with NYHA class I–III caused by coronary heart disease (54 males and 21 females, with an average age of 61.5). The proportions of patients with NYHA classes I, II, and III were 20% (15 patients), 64% (48 patients), and 16% (12 patients), respectively. All patients developed CHF due to coronary heart disease, with an average disease duration of 46.0 months (from 16 to 96 months). All patients received standard therapy: beta-blockers, angiotensin-converting enzyme inhibitors or angiotensin II receptor antagonists, diuretics, and statins; 25% of patients received nitrates, and 92.6% of patients received antiplatelet therapy (Table 1).

According to the 6-minute walk test, there was a significant reduction in physical activity tolerance in patients (the average distance was 385.5 m). Morphological and functional changes in cardiac parameters were sufficiently pronounced in all patients (with an average left ventricular ejection fraction of 38.2%). Complaints analysis and physical examination revealed that 80% of CHF patients (60 patients) had NYHA II-III prior to the study. According to the 6-minute walk test, the average distance was 385.5 m (354.0; 407.5), which corresponds to the a reduction in physical activity tolerance in patients with NYHA class II (Table 1). According to the echocardiographic data, the average left ventricular ejection fraction (LVEF) was 38.2% (33.4; 43.1). The average NT-proBNP plasma level was 240.3 pg/mL (70.83; 524.32), which corresponded to a normal level of this peptide in CVD patients (the plasma level

Table 1. Demographic characteristics of the study population

Number of patients	75
Males/females, n (%)	54 (72%); 21 (28%)
Average age (years)	61.5 (range 56-63)
CHF duration (months)	46.0 (24; 79)
NYHA classes I/II/III, n (%)	15 (20)/ 48 (64)/ 12 (16)
Coronary heart disease, n (%)	75 (100)
Previous myocardial infarction, n (%)	75 (100)

Table 2. Clinical parameters of the study population

Left ventricular ejection fraction, %	38.2 (33.4; 43.1)
Average 6-minute walk test distance, m	385.5 (354.0; 407.5)
CoQ10 plasma levels, ng/mL	826.3 (510.8; 1080.3)
NT-proBNP average plasma level, pg/mL	240.3 (70.83; 524.32)
Brachial artery diameter at rest, cm	0.51 (0.43; 0.56)

Table 3. Brachial artery diameter during reactive hyperaemia according to ultrasound images

	Normal vasodilatation following reactive hyperaemia	Moderate endothelial dysfunction	Significant endothelial dysfunction
Number of patients, n (%)	12 (16%)	40 (53.3%)	23 (30.7%)
Initial diameter of the brachial artery at rest, cm	0.57 (0.54; 0.59)	0.56 (0.55; 0.59)	0.57 (0.53; 0.60)
Diameter of the brachial artery following reactive hyperaemia, cm	0.65 (0.59; 0.66)	0.59 (0.57; 0.60)	0.58 (0.56; 0.61)
Brachial artery diameter increase, %	12.5	7.3	2.6
CoQ10 concentration, ng/Ml	1451.3 (1106.2; 1593.8)	1051.7 (702.5; 1239.0)	904.6 (678.2; 1027.5)
Percentage of patients with CoQ10 concentration of less than 700 ng/mL	41.7	45	52.2
NT-proBNP, pg/mL	216.8 (139.2; 404.5)	232.1 (153.4; 472.7)	283.1 (192.6; 523.8)
NYHA class	2.1	2.5	2.7
Ejection fraction, %	43.2 (39.2; 44.1)	40.0 (37.4; 42.9)	38.4 (35.7; 42.3)

was higher than that in healthy persons (0 – 125 pg/mL)). The initial average CoQ10 concentration was 826.3 ng/mL in all patients.

Association of the Doppler evaluation of endothelial-dependent vasodilatation and the clinical, morphological, and functional features of CHF patients

According to the Doppler evaluation of the brachial artery diameter, the initial diameter of the brachial artery was 0.51 cm (Table 3). The results of the Doppler evaluation of changes in the brachial artery diameter during reactive hyperaemia showed that only 12 patients (16%) had a proper increase in brachial artery diameter after the test and 63 patients (84%) had endothelial dysfunction. Additionally, 40 patients (53.3%) had moderate endothelial dysfunction with an increase in the brachial artery diameter from 5 to 10%, and 23 patients (30.7%) had severe endothelial dysfunction with an increase in the brachial artery diameter of less than 5%.

Taking into account that CoQ10 deficiency causes endothelial dysfunction; endothelial-dependent vasodilatation was assessed in all patients. Correlating the results of the ultrasound images of the brachial artery diameter following reactive hyperaemia and the CoQ10 concentration revealed that, poor endothelial-dependent vasodilatation caused by endothelial dysfunction in patients with CHF was accompanied by a reduction in the plasma CoQ10 concentration. There was evidence that the lower the CoQ10 concentration the higher the severity of endothelial dysfunction. Moreover, a correlation between the severity of endothelial dysfunction and the clinical morphological and functional characteristics was noted. Patients with a normal brachial artery diameter following reactive hyperaemia vasodilation had a higher level of the left ventricular ejection fraction (43.2% against 40.5 or 38.4%) and lower level of the average NYHA class (2.1 against 2.5 and 2.7) than patients with moderate to significant endothelial dysfunction (the differences were not significantly reliable). Moreover, the higher the level of endothelial dysfunction, the lower the LVEF and higher the NYHA class. Similar features were also observed when comparing the NT-proBNP plasma concentration with the severity of endothelial dysfunction: patients who were normal following reactive hyperaemia vasodilatation had a lower average NT-proBNP concentration (216.8 (139.2; 404.5)) than patients with moderate (232.1 (153.4; 472.7)) and significant (283.1 (192.6; 523.8)) endothelial dysfunction (Table 3).

Association of the NT-proBNP and the clinical, morphological, and functional features of CHF patients

The average NT-proBNP plasma concentration was 240.3 pg/mL (70.83; 524.32); nevertheless, the scatter of values was significant (from 20.2 to 860.0 pg/mL). To analyse the clinical, morphological, and functional features of CHF patients with different plasma levels of NT-proBNP, two groups of patients were identified: the first group consisted of CHF patients with NT-proBNP plasma levels of less than 125 pg/mL; the second group consisted of CHF patients with NT-proBNP plasma levels of more than 125 pg/mL. The characteristics of CHF patients that depended on the NT-proBNP plasma levels are shown in *Table 4*.

Table 4. Characteristics of CHF patients that depend on the NT-proBNP plasma levels

	CHF patients with NT-proBNP plasma levels less than 125 pg/mL	CHF patients with NT-proBNP plasma levels more than 125 pg/mL	*p*
Number of patients, n (%)	15 (20)	60 (80)	-
Average age, years	64.5 (59.0; 66.5)	63.0 (59.5; 69.0)	*0.98*
Males/females, n (%)	9(60) / 6(40)	45 (80) / 15 (20)	*0.48*
Average NT-proBNP plasma level, pg/mL	62.2 (49.8; 104.4)	264.16 (177.4; 491.9)	*0.001*
Average distance according to the 6-minute walk test, m	405.0 (393.0; 427.0)	336.5 (300.75; 408.0)	*0.079*
LVEF, %	42.7 (39.5; 44.3)	38.2 (36.3; 43.1)	*0.001*
Average CoQ10 plasma level, ng/mL	1020.0 (744.4; 1217.0)	631.6 (403.2; 972.5)	*0.228*

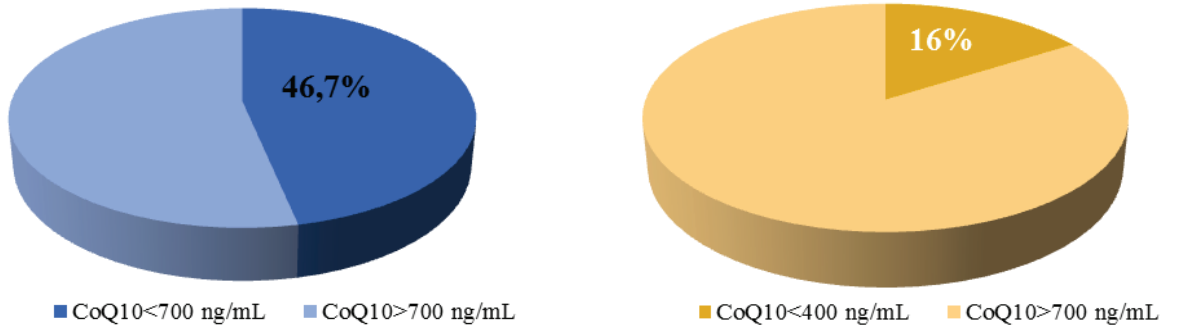


Figure 1. Percentage of CHF patients with low CoQ10 plasma levels

The difference in patient age was not significantly reliable, but the first group was characterized by a significantly higher number of women than the second (40% against 20%). All patients from the first group were diagnosed with CHF I NYHA class and had an average walk distance of 450.0 meters (393.0; 427.0) according to the 6-minute walk test. In the second group, the average distance according to the 6-minute walk test (336.5 meters) was less than the average distance recorded for the first group, and (300.8; 408.3) (p=0.079), which corresponded to an average distance according to the 6-minute walk test in patients with NYHA classes II and III. Significant differences were observed in the contractile function of the left ventricle (p=0.001): there was a significantly higher level of systolic function in the first group (LVEF 42.7 (39.5; 44.3)) and lower level in the second group (LVEF 38.2 (36.3; 43.1)) (p=0.001), according to the echocardiography results. No significant differences were observed in the CoQ10 plasma levels; however, there was a very slight trend towards significance: the average CoQ10 plasma level was 1020.0 ng/mL (744.4; 1217.0) in the first group and 631.6 n/mL (403.2; 972.5) in the second group (p=0.228).

Association of the coenzyme Q10 plasma concentration and the clinical, morphological, and functional features of CHF patients

In this study, we found a wide variety of CoQ10 plasma levels in CHF patients: 350 ng/mL to 1903 ng/mL. However, 35 patients (46.7%) had CoQ10 plasma levels less than 700 ng/mL, and 12 patients (16%) had CoQ10 plasma levels that were even lower (less than 400 ng/mL) (Fig. 1).

To analyse the clinical, morphological, and functional features of CHF patients with normal and low CoQ10 plasma levels, two groups of patients were identified. The first group consisted of CHF patients with CoQ10 plasma levels lower than 700 ng/mL (the average concentration was 513.1 ng/mL (442.5; 619.5)). The second group consisted of CHF patients with CoQ10 plasma levels higher than 700 ng/mL (the average concentration was 1042.0 ng/mL (960.0; 1283.5); p=0.001). The analysis of the CHF patient clinical and demographic features of CHF patients with initially normal and low CoQ10 plasma levels showed that there was a variety of non-significant differences in these groups (*Table 5*). There were more males than females in both groups (with no significant differences), and patients with lower CoQ10 plasma concentrations were younger than patients with normal CoQ10 plasma concentrations (there was a slight trend towards significance).

Patients with lower CoQ10 plasma levels had less CHF history and higher NYHA class (but these differences were not significant). Patients in this group had a more severe course of chronic heart failure: the average distance according to the 6-minute walk test was significantly lower (there was a trend towards significance) than average the distance in patients with normal CoQ10 plasma levels. Compared to patients with normal CoQ10 plasma levels, patients with lower CoQ10 plasma levels had non-significant higher NT-proBNP plasma levels and lower LVEFs.

Table 5.CHF patient characteristics depending on CoQ10 plasma levels

	CoQ10<700 ng/mL	CoQ10>700 ng/mL	*p*
Number of patients, n (%)	35 (46.7)	40 (53.3)	-
Average age, years	58.0 (54.0; 66.0)	63.0 (60.5; 70.0)	*0.18*
Males/females, n (%)	24(68.6) / 11(31.4)	30 (75) / 10 (25)	*0.82*
Average NT-proBNP plasma level, pg/mL	156.4 (121.2; 220.4)	105.68 (53.7; 240.7)	*0.221*
Average distance according to the 6-minute walk test, m	336.5 (307.3; 387.5)	405.0 (340.5; 432.0)	*0.059*
LVEF, %	39.5 (37.5; 43.0)	42.0 (40.5; 44.0)	*0.58*
Average CoQ10 plasma level, ng/mL	513.1 (442.5; 619.5)	1042.0 (960.0; 1283.5)	*0.001*

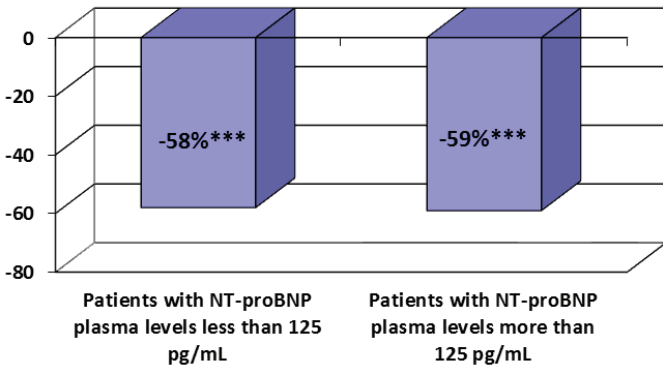


Figure 2. NT-proBNP plasma level dynamics under the influence of CpQ10-combined therapy in patients with NYHA class I-III

Association of the NT-proBNP plasma levels and the clinical, morphological, and functional features of CHF patients after the coenzyme Q10 treatment

According to the study design, patients started to receive CoQ10 therapy (60 mg/day) in addition to standard therapy for CHF after an initial CoQ10 plasma level evaluation. The use of CoQ10-combined therapy normalized the NT-proBNP plasma levels in both groups of patients (Figure 2).

Furthermore, CoQ10-combined therapy caused a significant increase in the average distance according to the 6-minute walk test (the average distance was slightly higher in patients with an initially increased NT-proBNP plasma level) and a non-significant LVEF increase (Table 6). An increase in CoQ10 plasma levels in both groups of patients was also observed (the dynamics were greater in patients with initially normal NT-proBNP plasma levels) (Table 6).

Association of the CoQ10 plasma levels and the clinical, morphological, and functional features of CHF patients after the coenzyme Q10 treatment

Some differences in the dynamics of the clinical, morphological, functional features, and laboratory test results in patients with different initial CoQ10 plasma levels were also observed. A significant reduction of the NT-proBNP plasma levels in both groups of patients was observed (the NT-proBNP plasma level was slightly higher in patients with initially higher CoQ10 plasma levels). There was evidence that the efficacy of CoQ10 therapy (Qudesan) was higher in patients with initially lower CoQ10 plasma levels (the dynamics of the 6-minute test results andas well as the LVEFs and CoQ10 plasma levels were greater in this group) (Table 7).

DISCUSSION

This prospective clinical study examined the relationships among the clinical, morphological, functional cardiac parameters, indicators of endothelial function, NT-proBNP plasma levels and CoQ10 concentration in patients with chronic heart failure of ischaemic aetiology.

According to this study, a wide range of CoQ10 concentrations was revealed: the CoQ10 plasma levels varied from 350 ng/mL to 1903 ng/mL and the average concentration was 826.3 (510.8; 1080.3) ng/mL. It was also shown that low levels of CoQ10 were associated with more severe heart failure and higher levels of NT-proBNP. Additionally, we revealed that high levels of NT-proBNP were associated with low exercise tolerance, reduced LVEF and a decrease in CoQ10 plasma levels.

Table 6. CoQ10-combined therapy (Qudesan) clinical, morphological, and functional features in patients with different NT-proBNP plasma levels

	Patients with NT-proBNP plasma levels less than 125 pg/mL (n=15)	Patients with NT-proBNP plasma levels more than 125 pg/mL (n=60)	*p*
Average NT-proBNP plasma level, pg/mL			
Before therapy	62.2 (49.8; 104.4)	264.2 (177.4; 491.9)	*0.001*
After 12 weeks	26.4 (13.6; 42.8)	108.20 (59.9; 155.8)	*0.001*
D%; p	-58; 0.005	-59; 0.001	
Average distance according to the 6-minute walk test, m			
Before therapy	405.0 (393.0; 427.0)	336.5 (300.8; 408.0)	*0.079*
After 12 weeks	457.0 (432.0; 520.0)	398.5 (351.3; 512.0)	*0.085*
D%; p	+12.8; 0.003	+18.4; 0.002	
LVEF, %			
Before therapy	43.0 (39.8; 44.0)	39.0 (36.5; 42.0)	*0.001*
After 12 weeks	46.0 (43.5; 49.0)	41.5 (39.5; 43.0)	*0.028*
D%; p	+6.9; 0.416	+6.4; 0.752	
Average CoQ10 plasma level, ng/mL			
Before therapy	1020.0 (744.4; 1217.0)	837.3 (510.8; 1080.3)	*0.228*
After 12 weeks	2310.0 (1160.0; 3415.0)	1000.0 (752.8; 1905.0)	*0.063*
D%; p	+126.5; 0.021	+19.4; 0.208	

Table 7. Changes in the CoQ10 plasma levels depending on, the dynamics of the clinical, morphological, and functional features, NT-proBNP and CoQ10 plasma levels

	Patients with CoQ10 plasma levels less than 700 ng/mL (n=35)	Patients with CoQ10 plasma levels more than 700 ng/mL (n= 40)	*p*
Average NT-proBNP plasma level, pg/mL			
Before therapy	156.4 (121.2; 220.4)	105.7 (53.7; 240.7)	*0.221*
After 12 weeks	87.5 (34.6; 277.4)	43.5 (15.2; 78.2)	*0.386*
D%; p	*-44.1; 0.046*	*-58.8; 0.001*	
Average distance according to the 6-minute walk test, m			
Before therapy	336.5 (307.3; 387.5)	405.0 (340.5; 432.0)	*0.059*
After 12 weeks	395.0 (339.5; 479.0)	453.0 (404.5; 525.0)	*0.101*
D%; p	*+17.4; 0.028*	*+11.8; 0.001*	
LVEF, %			
Before therapy	38.5 (35.0; 41.5)	41.0 (38.0; 43.5)	*0.58*
After 12 weeks	44.0 (42.0; 46.0)	42.0 (39.0; 45.0)	*0.78*
D%; p	*+14.3; 0.144*	*+2.4; 0.609*	
Average CoQ10 plasma level, ng/mL			
Before therapy	513.1 (442.5; 619.5)	1042.0 (960.0; 1283.5)	*0.001*
After 12 weeks	965.5 (545.5; 2410.0)	1910.0 (1365.0; 2460.0)	*0.208*
D%; p	*+88.2; 0.144*	*+83.2; 0.016*	

The study results indicate that manifested by impaired endothelium-dependent vasodilatation, endothelial dysfunction is accompanied by a reduction in the CoQ10 plasma level in patients with CHF. Additionally, the lower the CoQ10 plasma level the more severe the endothelial dysfunction. Therefore, it is expected that a correction of the CoQ10 deficiency will be required. CoQ10 deficiency correction will improve the endothelial function, thus reducing the tissue hypoxia associated with CHF. Furthermore, other study results showed that a reduction of the CoQ10 concentration to less than 730 ng/mL, was a poor prognostic factor in CHF patients. It is argued that a low CoQ10 plasma level is an independent mortality risk factor in this group of patients (22-25). This study revealed that the optimal CoQ10 concentration for the prediction of mortality was 730 ng/mL. Multivariate analysis, in which the concentration of CoQ10 was compared with standard predictors of survival in CHF patients (age, sex, history of myocardial infarction, NT-proBNP concentration (terminal pro b-type natriuretic peptide)), and the glomerular filtration rate (with the modification of diet in renal disease) showed that CoQ10 was an independent predictor of survival in CHF patients (23-26).

The literature suggests that lower CoQ10 levels are associated with an increasing severity of HF symptoms. Additionally, the aforementioned evidence suggests that CoQ10 may be useful in patients with CHF by replenishing deficient levels, which may improve ATP synthesis and left ventricular function. The beneficial effects of coenzyme Q10 supplementation have been observed in several age-related diseases, including heart failure. The CoQ10 (coenzyme Q10) level is significantly decreased in patients with this disease, which correlates with the severity of clinical symptoms (30).

To clearly that that the CoQ10 levels are corrected with the severity of clinical symptoms, we used a 12-wk treatment of CoQ10 in patients with HF. Our experience with CoQ10 therapy in patients with class II-III NYHA provide us with hope of success. We previously determined the most effective dose for patients with CHF (24-28). The soluble form of the drug was used as the CoQ10 therapy (20 mL vials), 1 mL of which contains 30 mg of CoQ10 and 4.5 mg of Vitamin E. CHF patients received different doses of CoQ10: 60 mg (2 mL of Qudesan® solution), 90 mg (3 mL of Qudesan® solution), and 120 mg (4 mL of Qudesan® solution). After 4 weeks of treatment, it was found that regardless of the CoQ10 dose, there was more a 2-fold increase in the CoQ10 concentration in all patients with CHF. In accordance with the data, we determined that the optimal daily CoQ10 dose of combination therapy in patients with CHF was 60 mg per day.

Some foreign studies used much higher doses of CoQ10 (18), because the liposoluble form of the drug was used. Qudesan® solution is a solubilized form of CoQ10, the bioavailability of which is higher than the bioavailability of the liposoluble form by at least 2.6 times. Therefore, a dose of 60 mg of water-soluble (solubilized form) CoQ10 should be approximately equal to 150-200 mg of liposoluble CoQ10.

It was found that treatment with CoQ10 led to more significant dynamics of the clinical, morphological, and functional parameters (the dynamics of the average distance according to the 6-minute walk test, LVEFs, and CoQ10 plasma levels) in patients with initially lower levels of CoQ10. We also also showed that the addition of CoQ10 to standard therapy for CHF patients with initially high NT-proBNP concentrations and reduced CoQ10 concentrations led to a significant decrease in the NT-proBNP plasma levels, increase in the CoQ10 concentrations, in-

crease in exercise tolerance and improvement of endothelial function.

There have been numerous observational reports over the last few decades reporting the usefulness of CoQ10 for improving HF symptoms, including the ejection fraction, left ventricular size and quality of life. However, these studies had several design shortcomings that prevented their translation into clinical practice (18, 31).

Our findings on the effect of CoQ10 in CHF combination therapy on the BNP and CoQ10 plasma levels are consistent with those of other authors. Tokareva et al. showed that long-term use of CoQ10 prevented the progression of chronic heart failure in patients with impaired myocardial contractility and original BNP levels in the normal range (less than 100 pg/mL) (29). In another analysis by Sander et al., with CoQ10 doses ranging from 60–200 mg/day, it was shown that there was a 3.7% net improvement in the ejection fraction (1.59 to 5.77; p<0.00001) (17).

Fotino et al. reported similar results in a meta-analysis; CoQ10 supplementation resulted in a pooled mean net increase in the ejection fraction of 3.67%, further suggesting the benefits of CoQ10 therapy (32).

The largest randomized trial performed to date (completed in 1993 and enrolled 641 patients) demonstrated that compared with placebo, CoQ10 reduced the risk of HF hospitalization (73 versus 118, $p<0.001$) and complications of HF, such as pulmonary oedema and cardiac asthma (20 versus 51 and 97 versus 198, $p<0.001$), and our results are in accordance with these findings (33).

Coenzyme Q10 potentially enhances cardiac function, probably through a variety of mechanisms. CoQ10 plays a critical role in ATP generation by accepting electrons from complexes I and II and transporting them to complex III, and at which point they are ready to be reduced by complexes I and II again. This electron transportation allows hydrogen ions (H^+) to be pumped across the inner mitochondrial membrane (IMM), which drives the synthesis of ATP via ATP synthase. CoQ10 has been shown to inhibit the peroxidation of cell membrane lipids and reduce the oxidation of circulating lipolipids. In addition to its antioxidant activity, CoQ10 also seems to improve endothelial function (34, 35).

CONCLUSION

In HF patients, CoQ10 supplementation induced improvements in functional cardiac parameters, such as the ejection fraction. Our results suggest that al CoQ10 supplementation may be a useful option for effective management of heart failure and warrant future adequately powered randomized controlled trials of CoQ10 supplementation in patients with HF.

Conflict Of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgements

The determination of CoQ10 plasma levels and the writing of the article were supported by Grant of Russian Science Foundation No. 14-15-00126.

REFERENCES

1. Go AS, Mozaffarian D, Roger VL, et al. Heart disease and stroke statistics 2014 update: a report from the American Heart Association. American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Circulation 2014; 129: 280-292.
2. Ho KK, Pinsky JL, Kannel WB, et al. The epidemiology of heart failure: the Framingham Study. J Am Coll Cardiol 1993; 22: 6–13.
3. McMurray JJ, Adamopuulus S, Anker SD, et al. ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure 2012: The Task Force for the Diagnosis and Treatment of Acute and Chronic Heart Failure 2012 of the European Society of Cardiology Deloped in collaboration with the Heart Failure Association (HFA) of the ESC. Eur Heart J 2012; 33: 1787–847.
4. Yancy CW, Jessup M, Bozkurt B, et al. ACCF/AHA Guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association task force on practice guidelines. Circulation 2013; 128: 240-327.
5. Aronov DM. Importance of coenzyme Q10 in cardiology. Russian Medical Journal, 2007; 15: 1484-1488.
6. Fotino AD, Thompson-Paul AM, Bazzano LA. Effect of coenzyme Q10 supplementation on heart failure: a meta-analysis. Am J Clin Nutr 2013; 97: 268–275.
7. Mortensen SA. Coenzyme Q10: will this natural substance become a guideline-directed adjunctive therapy in heart failure? JACC Heart Fail 2015; 3:270-271.
8. Miles MV, Horn PS, Tang PH, et al. Age-related changes in plasma coenzyme Q10 concentrations and redox state in apparently healthy children and adults. AJ Clin Chim Acta 2004; 347: 139-144.
9. Medvedev OS, Kalenikova EI, Gorodetzkaya EA, et al. Coenzyme Q10 in cardiology practice – theoretical bases and clinical studies results. Russian Medical Journal, 2009; 17: 1177-1181.
10. R. Ferrari, L. Agnoletti, L. Comini, G. et al. Oxidative stress during myocardial ischemia and heart failure, Eur Heart J 1998; 19:2–11.
11. Sarah L. Molyneux, Christopher M, et al. Coenzyme Q10 An Independent Predictor of Mortality in Chronic Heart Failure Christchurch, New Zealand. J Am Coll Caqrdiol 2008; 52: 1435-1441.
12. Madamanchi NR, Runge MS. Mitochondrial dysfunction in atherosclerosis. Circ Res. 2007; 100: 460–473.
13. Ponikowski P, Voors AA, Anker SD et al. 2016 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: The Task Force for the diagnosis

and treatment of acute and chronic heart failure of the European Society of Cardiology. Developed with the special contribution of the Heart Failure Association (HFA) of the ESC. Eur Heart J 2016, 20.
14. Berezin AE. Biological markers in chronic heart failure: hopes, reality, perspectives. Serceva nedostatnist 2013; 1:5-15.
15. Tsutamoto T. Plasma brain natriuretic peptide level as a biochemical marker of morbidity and mortality in patients with asymptomatic or minimally symptomatic left ventricular dysfunction. Comparison with plasma angiotensin II and endothelin-1. Eur Heart J 199; 20: 1799-1807.
16. Soja AM, Mortensen SA. Treatment of congestive heart failure with coenzyme Q 10 illuminated by meta-analyses of clinical trials. Mol Aspects Med 1997; 18: 159–168.
17. Sander S, Coleman CI, Patel AA, et al. The impact of coenzyme Q10 on systolic function in patients with chronic heart failure. J Card Fail 2006; 12: 464–472.
18. Judy WV, Hall JH, Toth PD, et al. Double blind-double crossover study of coenzyme Q10 in heart failure, in: Biomedical and Clinical Aspects of Coenzyme Q, Vol. 5. K. Folkers and Y. Yamamura, eds, Elsevier, Amsterdam 1986, pp. 315–323.
19. Munkholm H, Hansen HHT, Rasmussen K. Coenzyme Q10 treatment in serious heart failure. Ann Intern Med 1999; 9: 285–289.
20. Wolsk E, Claggett B, Pfeffer MA, et al. Role of B-Type Natriuretic Peptide and N-Terminal Prohormone BNP as Predictors of Cardiovascular Morbidity and Mortality in Patients With a Recent Coronary Event and Type 2 Diabetes Mellitus. J Am Heart Assoc. 2017; 6: 43-47.
21. Edlund PO. Determination of coenzyme Q10, α-tocopherol and cholesterol in biological samples by coupled-column liquid chromatography with coulometric and ultraviolet detection. J Chromatogr 1988; 425: 87–97.
22. Rosenfeldt FL, Haas SJ, Krum H, et al. Coenzyme Q10 in the treatment of hypertension: a meta-analysis of the clinical trials. J Hum Hypertens 2007; 21:297-306.
23. Gorokhova SG, Sizova ZM. Pharmacotherapy of chronic heart failure in ambulatory practice. Physician's workbook. Medpraktika-M 2008; pp.85.
24. Mortensen SA, Rosenfeldt F, Kumar A, et al. The effect of coenzyme Q10 on morbidity and mortality in chronic heart failure: results from Q-SYMBIO: a randomized double-blind trial. JACC Heart Fail. 2014; 2:641–649.
25. Keogh A, Fenton S, Leslie C, et al. Randomized double-blind, placebo-controlled trial of coenzyme Q10 therapy in class II and III systolic heart failure. Heart Lung Circ. 2003; 12:135–141.
26. DiNicolantonio JJ, Bhutani J, McCarty MF, et al. Coenzyme Q10 for the treatment of heart failure: a review of the literature. Open Heart 2015; 2: 32-36.
27. Alehagen U, Johansson P, Bjornstedt M, et al. Cardiovascular mortality and N-terminal-proBNP reduced after combined selenium and coenzyme Q10 supplementation: a 5-year prospective randomized double-blind placebo-controlled trial among elderly Swedish citizens. Int J Cardiol. 2013; 167:1860-1866.
28. Sizova ZM, Farafoniva TN, Medvedev OS, et al. Possibilities of different doses coenzyme Q10 administration in patients with chronic heart failure. Heart failure 2010; 11: 218-222.
29. Tokareva OG, Kharitonova EV, Mareev VY, et al. Vliyanie koenzima Q10 v sostave standartnoj terapii bol`ny`x xronicheskoj serdechnoj nedostatochnost`yu na plazmenny`e urovni mozgovogo natrijureticheskogo peptida. Zhurnal Serdechnaya Nedostatochnost 2014; 850: 232–237.
30. Jankowski J, Korzeniowska K, Cieślewicz A, et al. Coenzyme Q10 - A new player in the treatment of heart failure? Pharmacol Rep 2016; 68: 1015-1019.
31. Lampertico M, Comis S. Italian multicenter study on the efficacy and safety of coenzyme Q10 as adjuvant therapy in heart failure. Clin Investig. 1993; 71: 129-133.
32. Fotino AD, Thompson-Paul AM, Bazzano LA. Effect of coenzyme Q_{10} supplementation on heart failure: a meta-analysis. Am J Clin Nutr 2013; 97: 268-75.
33. Morisco C, Trimarco B, Condorelli M. Effect of coenzyme Q10 therapy in patients with congestive heart failure: a long-term multicenter randomized study. Clin Investig. 1993; 71: 134–136.
34. Crane FL, Hatefi Y, Lester RL, et al. Isolation of a quinone from beef heart mitochondria. Biochim Biophys Acta 1957; 25: 220–1.
35. Mellors A, Tappel AL. The inhibition of mitochondrial peroxidation by ubiquinone and ubiquinol. J Biol Chem 1966; 241: 4353–4356.

EFFICACY OF GENITAL CHLAMIDIAE TRACHOMATIS TREATMENT IN WOMEN OF REPRODUCTIVE AGE

Aleksandra Dimitrijevic, Zoran Protrka, Nikola Jovic, Petar Arsenijevic
Department of Gynecology and Obstetrics, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
Clinic of Obstetrics and Gynecology, Clinical Center Kragujevac, Kragujevac, Serbia

EFIKSANOST TERAPIJE GENITALNIH INFEKCIJA HLAMIDIJOM TRAHOMATIS KOD ŽENA U REPRODUKTIVNOM PERIODU

Aleksandra Dimitrijević, Zoran Protrka, Nikola Jović, Petar Arsenijević
Katedra za ginekologiju i akušerstvo, Fakultet medicinskih nauka Univerziteta u Kragujevcu, Kragujevac, Srbija
Klinika za ginekologiju i akušerstvo, Klinički centar Kragujevac, Kragujevac, Srbija

Received / Primljen: 29. 11. 2016. Accepted / Prihvaćen: 30. 11. 2016.

ABSTRACT

Cervicitis is inflammation of the cervix, and the causes of such inflammation may include infection from certain sexually transmitted diseases (STDs), injury to the cervix from a foreign body inserted into the vagina (for example, birth control devices such as a cervical cap or diaphragm), or cervical cancer, whose course can be subacute or chronic. Our research aimed to test the efficacy of the proposed treatment protocol for chlamydia trachomatis distal genital infections in reproductive women. This single-centre, randomized, quasi-experimental prospective study was conducted among 40 women with diagnosed Chlamydia Trachomatis (CT) cervical infections who were diagnosed and treated at the Clinic of Obstetrics and Gynaecology in the Clinical Center Kragujevac in Serbia from December 2014 to January 2015. Patients were divided into two groups according to the treatment method: the tetracycline group (n=20), with doxiciclyn (Dovicin®) given at a dose of 100 mg twice per day for 10 days and 100 mg per day for the next 10 days, and the macrolides group (n=20), with azithromycin (Hemomycin®) at a dose of 1000 mg per day, divided into four doses or a single dose per day. Treatment with doxycycline proved to be statistically more effective compared to treatment with azithromycin. Our results confirm that the outcome of infections caused by C. trachomatis depends solely on the applied therapy and management, but extensive prospective studies in a female cohort that includes more parameters, such as potential age related, dose-dependent and adherence variability, are necessary to determine and confirm the best choice for treatment of CT cervicitis.

Keywords: *Chlamydia Trachomatis, cervicitis, Tetracyclines, Macrolides*

SAŽETAK

Cervicitis je bolest od koje oboli oko 50% svih žena, a najčešći izazivač je Chlamidia trachomatis.Chlamidia trachomatis je i najčešća seksualno prenosiva bolest.

Prospektivna studija sprovedena u klinici za Ginekologiju i akušerstvo obuhvatila je 40 pacijentkinja kod kojih je analizom cervikalnog brisa utvrđeno prisustvo Chlamidiae trachomatis nakon čega su one lečene različitim terapijskim protokolima i pri tome ispitivana efikasnost terapije.Prema terapiskom protokolu pacijentkinje su bile podeljene u dve grupe. Prva grupa u kojoj je bilo 20 pacijentkinja lečena je tetraciklinima,a druga grupa makrolidnim antibioticima.

Analizom dobijenih rezultata utvrđeno je da je u grupi pacijentkinja lečenih tetraciklinima po završenoj terapiji Chlamidia trachomatis bila prisutna u brisu cerviksa kod 15% pacijentkinja, u 85% slučajeva nalaz je bio negativan. U grupi pacijentkinja lečenih makrolidima pozitivan nalaz Chlamidiae trachomatis zabeleženje u 85% slučajeva, a samo 15% pacijentkinja po završenoj terapiji nije više imalo Chl. Trachomatis u cervikalnom brisu.

Lečenje tetraciklinima pokazalose statistički značajno efikasnije u odnosu na lečenje makrolidnim antibioticima. Prema tome i drugim rezultatima naseg istrazivanja, terapija izbora za lecenje Chlamidiae trachomatis treba da bude doksiciklin.

Ključne reči: *Chlamydia trachomatis, cervicitis, inflamacija grlića materice, tetraciklini, makrolidi*

sciendo

UDK: 616.98-085.33:579.882 / Ser J Exp Clin Res 2018; 19 (2): 151-157
DOI: 10.1515/SJECR-2016-0092

Corresponding author: Aleksandra Dimitrijevic,
Department of Gynecology and Obstetrics, Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia,
e-mail: adimitrijevic1@hotmail.com

INTRODUCTION

Cervicitis is inflammation of the cervix, and the causes of such inflammation include infection from certain sexually transmitted diseases (STDs), injury to the cervix from a foreign body inserted into the vagina (for example, birth control devices such as a cervical cap or diaphragm), or cervical cancer (1, 2) whose course can be subacute or chronic.

Infectious cervicitis may be caused by Chlamydia trachomatis (CT), Neisseria gonorrhoeae or herpes simplex virus (HSV), but the inciters may be bacteria, viruses and fungi. In smears of the cervixes of women who were diagnosed with cervicitis, the presence of the Gram-negative obligate intracellular bacteria Chlamydia trachomatis found in approximately 20 % of the cases, and it is considered the most common cause of Chlamydia trachomatis cervicitis (3-5).

According to the sexually transmitted diseases treatment guidelines from 2010, two major diagnostic signs characterize cervicitis: 1) a purulent or mucopurulent endocervical exudate visible in the endocervical canal or on an endocervical swab specimen (commonly referred to as mucopurulent cervicitis or cervicitis) and 2) sustained endocervical bleeding that is easily induced by gentle passage of a cotton swab through the cervical os. Either or both signs might be present. Cervicitis is frequently asymptomatic, but some women complain of an abnormal vaginal discharge and intermenstrual vaginal bleeding (e.g., after sexual intercourse). A finding of leucorrhoea (>10 WBC per high-power field on microscopic examination of vaginal fluid) has been associated with chlamydial and gonococcal infections of the cervix. In the absence of inflammatory vaginitis, leucorrhoea might be a sensitive indicator of cervical inflammation with a high negative predictive value (6).

The infection is very often asymptomatic; more than 70 % of women have no symptoms (7, 8). The infection, other than sexual contact with an infected partner, can be transmitted vertically if pregnancy occurs, i.e., in childbirth during the passage of the foetus through the birth canal of the infected mother. Newborn infections manifest clinically as oftalmia neonatorum in 50 % of cases or as pneumonia in 20 % of cases (9, 10).

The symptoms of cervicitis induced by C. trachomatis are vaginal discharge, pain in sexual intercourse and pain interference with defecation (11-13). The diagnosis of cervicitis induced by Chl. trachomatis typically is made with the following (14-19): isolation of the carrier in the respective cell culture; identification of the chlamydia antigen (DIF, immunosorbent assays, DNK hybridization, PCR and LCR); and serological tests for the detection of a specific antibody (response complements fixation, test indirect fluorescence, and ELISA-test).

When treating cervical infections caused by C. trachomatis, antibiotics are applied that penetrate well into the host cell because it is sensitive to antibiotics only in the reticular body during the process of division within the cytoplasm. Currently, this is considered the most effective method of treatment with antibiotics from the tetracycline and macrolide groups (20-22).

The Centers for Disease Control and Prevention (CDC) suggest that several factors should affect the decision to provide presumptive therapy for cervicitis or to await the results of diagnostic tests. Treatment with antibiotics for C. trachomatis should be provided for those women who are at an increased risk for this common STD (e.g., those aged ≤25 years, those with new or multiple sex partners, and those who engage in unprotected sex). The recommended regimens for presumptive treatment of cervicitis caused by CT is 1 g of azithromycin orally in a single dose or 100 mg of doxycycline orally twice a day for seven days (6).

Tetracyclines are naphthacene derivatives that inhibit protein synthesis and act bacteriostatic, and the macrolide inhibits protein synthesis and acts bacteriostatic.

Many follow-up studies have been conducted as recommended in different guidelines and according to empirical knowledge, but it is still unclear if doxycycline treatment is more efficacious than azithromycin in the management of cervicitis caused by Chlamydia.

Our research was aimed to test the efficacy of the proposed treatment protocol for Chlamydia trachomatis distal genital infections in women of reproductive age.

PATIENTS AND METHODS

Study design and setting

This single-centre, randomized, quasi-experimental prospective study was conducted among 40 women who were diagnosed with *Chlamydia Trachomatis* (CT) cervical infections and were diagnosed and treated at the Clinic of Obstetrics and Gynaecology in the Clinical Center Kragujevac in Serbia from December 2014 to January 2015.

Women ≥20 years of age who were confirmed to have a CT infection were divided into two groups according to the treatment method: Tetracyclines group and Macrolides group. The first group of patients was treated by tetracycline antibiotics (n=20), i.e., Doxiciclyn (Dovicin®), at a dose of 100 mg twice per day for 10 days and 100 mg per day for the next 10 days. The second group was treated by macrolide antibiotics (n=20), i.e., Azitromycin (Hemomycin®), at a dose of 1000 mg per day, divided into four doses or a single dose per day.

Detecting Chlamydia trachomatis (CT) Infections

In all patients, localized CT infections were examined by assays for direct pathogen detection, such as culture, antigen tests (direct fluorescent antibody (DFA), and immune chromatographic RDTs), nucleic acid hybridization and amplification tests as previously described (23). The established cell lines for isolating C. trachomatis include Mc Coy, HeLa 229 or Buffalo Green Monkey Kidney cells. Vaginal and endocervical swabs are suitable specimens for culture but must be collected using special devices and

transport media (24). Specimens were centrifuged onto a confluent cell monolayer and analysed for the development of characteristic intracytoplasmic inclusions after 48–72 h by staining with Giemsa, iodine, or fluorescence labelled antibodies to chlamydial antigens (LPS or MOMP). When using MOMP-specific antibodies for staining cell culture, the detection is highly specific, so this method has long been considered the reference test for CT detection (25).

During follow-up, samples were collected before and after antibiotic therapy from the endocervix and tested for CT.

Participant Selection

Inclusion criteria were the reproductive period of a woman who was diagnosed with CT cervicitis. Exclusion criteria were a previous allergic reaction to an antibiotic medicine or the same therapy applied with a partner. Additionally, clinical, biochemical, haematological and additional examinations were excluded for cardiovascular, neurological, endocrine and malignant conditions. To minimize bias, members of the test and control groups were fully comparable (matched-subjects). All participants provided written informed contest.

Treatment outcomes were cervicitis or vaginal symptoms and CT tests at follow-up.

All participants were asked to complete a questionnaire that included the following information: age, consumption of alcohol and cigarettes, age of first sexual contact, number of previous births and abortions, length of sexual life and number of sexual partners.

Statistical analyses

Descriptive statistics, hypothesis tests and logistic regression were included as statistical analyses methods. Measures of central tendency (arithmetic mean) and variability (SD, min, max) were used in descriptive statistics. Hypothesis testing was applied to the numerical insignia of the observation and analysis of the frequency of categorical variables. Before testing numerical variables, the type of distribution was assessed. For a normal distribution of data, a t-test and analysis of variance (ANOVA) were applied; in nonparametric statistics (distribution was not normal), a Mann-Whitney U-test was applied. To analyse the frequency of observed parameters, a Hi square test, Fisher's test and Mc Nemar's test were used, depending on the structure of data. In all hypothesis testing cases, the probability of the zero (null) hypothesis was $p \leq 0.05$. To estimate the importance of the impact of individual risk factors in relation to the dichotomous outcome, a logistic regression model, with univariate and multivariate regression analysis was used.

RESULTS

Demographic and anamnestic data

All women were in the range of 20 to 35 years old. In the doxycycline group, patients had fewer years of sexual relationships and fewer partners during their sexually active history. The frequencies of spontaneous and induced abortions were similar in both groups, as was that of deliveries. The use of contraception was more frequent in the azithromycin group compared to the doxycycline group, in which predominant use of condom contraception occurred occasionally and oral contraception occurred constantly (Table 1).

Table 1. Anamnestic data of all participants obtained from the questionnaires prior to follow-up. All data are presented as the mean±SD, minimum, maximum and/or frequencies in percent (%).

PARAMETERS		Doxycycline treatment	Azitromycine treatment
Year of sexual relations (X±SD)		15.25±2.51	18.35±2.06
Number of partners (min-max)		5 (2-8)	5-6 (3-7)
Spontaneous abortions	no	17 (85%)	17 (85%)
	two	3 (15%)	1 (5%)
	three	0 (0%)	1 (5%)
	four	0 (0%)	1 (5%)
Induced abortions	no	19 (95%)	19 (95%)
	two	0 (0%)	1 (5%)
	there	1 (5%)	0 (0%)
Deliveres	no	16 (80%)	16 (80%)
	one	1 (5%)	2 (10%)
	two	2 (10%)	2 (10%)
	theree	1 (5%)	0 (0%)
Contraception	Yes	3 (15%)	8 (40%)
	No	17 (85%)	12 (60%)
Type of contraception	condom contraception occasionally	3 (15%)	7 (35%)
	oral contraceptives	0 (0%)	1 (5%)

CT tests before and after doxycycline/azithromycin treatment

The outcomes of therapy are verified and cervical smears were compared before and after the therapy. Treatment with doxycycline proved to be statistically more effective compared to treatment with azithromycin. Based on the relative risk for a bad outcome, the efficacy of doxycycline therapy was 32 times higher than the effectiveness of treatment with azithromycin.

The cervical smear with Chlamydia trachomatis was significantly different before and after doxycycline treatment (Mc Nemar test; p=0.000). Before the therapy, all patients had a positive swab for cervix Chlamydia T. After therapy, a positive swab result was found only in 15 % of the respondents, and 85 % of the cervical swabs for Chlamydia were negative, as shown in Table 2.

Table 2. CT tests before and after doxycycline/azithromycin treatment. All data are presented as the mean±SD, minimum, maximum and/or frequencies in percent (%).

PARAMETERS			Doxycycline Treatment	Azitromycine treatment
Cervical smear on Chlamydia T.	Before therapy	positive	20 (100%)	20 (100%)
		negative	0 (0%)	0 (0%)
	After therapy	positive	3 (15%)	17 (85%)
		negative	17 (85%)	3 (15%)

Table 3. Results of univariate logistic regression analyses of different parameters among groups. Asterisk (*) represents statistical significance ($p<0.05$).

PARAMETERS	Regression coefficient (R)	Relative riskexp (B)=RR	Significance (p)
Overage age	0.11	0.902	0.102
Marital status	0.000	0.027	0.810
Age of sexual relations	-0.25	0.7	0.019*
Number of partners	0.000	1.042	0.839
Number of spontaneus abortions	0.000	1.189	0.617
Number of intended abortion	0.000	1.323	0.837
Number of births	0.000	1	0.989
Contraception	0.000	1.286	0.724
Type of contraception	0.000	1.185	0.887
Presence of clinical symptoms	0.000	0.643	0.568
Clinical symptoms	0.000	1	0.545
Diagnosis	0.000	1.489	0.412
Therapy	-0.490	0.031	0.0001*

Table 4. Results of multivariate logistic regression used for analysis of impact before treatment and treatment factors for results. Asterisk (*) represents statistical significance ($p<0.05$).

PARAMETERS	Regression coefficient (R)	Relative risk exp (B)=RR	Significance (p)
Age of sexual relations	0.000	0.988	0.953
Therapy	-0.387	0.0323	0.0013*

The Chlamydia findings in the cervix smear before and after implementation of azithromycin did not significantly differ (Mc Nemar test p=0.250). All respondents who were treated with this antibiotic before the application had a positive Chlamydia cervix smear. After therapy, 85 % of the patients had a positive smear finding, and 15 % of the patients who were positive before therapy had a negative smear.

Univariate logistic regression analyses among groups

The results of univariate logistic regression were used to analyse the impact of treatment factors on the treatment outcome. Univariate analysis showed that the years of sexual relations and type of treatment were statistically significant. Patients who had previously entered into their first sexual relations had a higher percentage of treatment failure, and their risk of bad outcome was 1.5 times higher. Treatment with doxycycline, in infections caused by chlamydia, is shown to be significantly more successful than treatment with azithromycin. Based on the relative risk for bad outcomes, which in this case is represented by a positive Chlamydia swab result and after the conducted treatment, therapy efficiency was 32 times higher than the treatment efficiency with azithromycin (*Table 3*).

Multivariate logistic regression analyses among groups

In the multivariate model, the two above-mentioned factors were statistically significant: age of sexual relations and applied therapy. Relative risk in the multivariate model showed that doxycycline in relation to azithromycin is 30 times more efficient in the treatment of infections caused by chlamydia (Table 4).

DISCUSSION

This single-centre, randomized prospective study was conducted among 40 women who were diagnosed with Chlamydia Trachomatis (CT) cervical infections to test the efficacy of the proposed treatment protocol for trachomatis Chlamydia genital infections in reproductive age women.

Chlamydia trachomatis, a small gram-negative bacterium, is the most common cause of bacterial sexually transmitted infections (STI) in both men and women (6). In the United States, it is the most commonly reported nationally notifiable disease (5-9). A significant proportion of patients are asymptomatic, thereby providing an ongoing reservoir for infection. The most frequent clinical manifestation of chlamydial infection in men is urethritis, and the most common finding in women is cervicitis. Thus, managing these conditions is essential.

Several factors should affect the decision to provide presumptive therapy for cervicitis.

Oral treatment with 100 mg of doxycycline taken twice daily for 7 days or with a single 1-g dose of azithromycin has been recommended since 1996 (6).

Previous research showed that there are significant differences in the efficiency of treating chlamydia trachomatis with doxycycline and azithromycin. Schwepeke and co-authors, in a large study in a male population diagnosed with Chl. trachomatis, showed that efficiency with doxy-

cycline was 95 % in relation to azithromycin (77 %) (26). Later, Kong and co-authors showed even less efficiency with doxycycline in relation to azithromycin than to the earlier study (27, 28). A metanalysis by Kong showed a minimal difference of 2-6 % with doxycycline in relation to azithromycin. In our study, only 15 % of patients had a positive cervical smear for chlamydia after therapy in the group treated with doxycycline.

The literature suggests a lower efficacy of azithromycin, but the reasons for this remain unclear. In groups with very similar demographic and anamnestic characteristics, differences between treated groups were significant (Tables 2 and 3). Univariate analysis showed that the year of sexual relations and the type of treatment were found to be significant. Patients who had previously entered in the first sexual relations had a higher percentage of treatment failure, and their risk of a poor outcome was 1.5 times higher. Treatment with doxycycline in infections caused by chlamydia is shown to be significantly more successful than is treatment with azithromycin. Based on the value of the relative risk for bad outcomes, which in this case is represented by a positive chlamydia swab result after treatment, the efficiency of the therapy was 32 times higher than the treatment efficiency with azithromycin (Table 3). One of the possible explanations of this result may be in the adherence of therapy in both groups. In fact, doxiciclyn (Dovicin®) was used in a dose of 100 mg twice per day for 10 days, whereas azithromycin (Hemomycin®) was used in a dose of 1000 mg per day, divided into four doses or a single dose per day. These differences in dosing type can be a limiting factor for therapy efficiency because of the lower adherence in the macrolides group.

Furthermore, the multivariate model showed that the two above-mentioned factors were statistically significant: age of sexual relations and applied therapy. The relative risk in the multivariate model showed that doxycycline in relation to azithromycin was 30 times more efficient in the treatment infections caused by chlamydia (Table 4).

Our results are completely in accordance with previous study results. Geisler et al concluded that in the context of a closed population receiving directly observed treatment for urogenital chlamydia infection, the efficacy of azithromycin was 97 %, and the efficacy of doxycycline was 100 % (38). Additionally, other studies have shown a lower efficacy for azithromycin compared with doxycycline. A meta-analysis of 23 randomized trials indicated that the efficacy of doxycycline was 3 percentage points higher than that of azithromycin for the treatment of urogenital chlamydia and was 7 percentage points higher than that of azithromycin for the treatment of symptomatic males; this resulted in renewed recommendations for trials to address the noninferiority of azithromycin (39).

In that sense, Lusk and coworkers concluded that empiric azithromycin treatment of cervicitis reduces cervicitis at follow-up in populations with a high prevalence of chlamydia trachomatis and/or mycoplasma genitalium, but there were no benefits of empiric azithromycin for non-specific cervicitis or empiric partner treatment (40).

These results suggest that there is a difference in the efficacy of the mentioned medicines, but we cannot dismiss the possibility that azithromycin can be effective in treating urogenital infection caused by CT as well as chlamydial cervicitis in women.

The literature describes therapy for cervical infection caused by chlamydia trachomatis as tetracycline and macrolide antibiotics, and the efficiency of these drugs based on results of most authors is almost equal (28-30). When we compared the dose-effective potential of these medications, the dose was almost empirically established. A previous study noted that 1000 mg of azithromycin was equally efficient in the treatment of chlamydia trachomatis as doxycycline applied in 200 mg doses per day for 7 days (31, 32).

Our study treatment protocol was a little different. We applied azithromycin in a single dose of 1000 mg and doxycycline at a dose of 100 mg for 10 days twice per day and later in a single dose. Our results show that doxycycline was significantly more efficient in the treatment of cervical infection caused by chlamydia trachomatis compared to azithromycin treatment. These differences in administration type did not influence the efficacy of the treatments separately, but generally, the frequency of oral intake of medicine can be a reason for lower adherence and, thus, indirectly for efficiency (33-35). In settings in which adherence is questionable, a single oral 1-g dose of azithromycin remains an excellent option because of the ease of its use and administration, in contrast to doxycycline.

However, treatment with azithromycin is almost half the price of treatment with doxycycline. Therefore, because of economic reasons, in some countries, the first choice of treatment for cervical infections caused by chlamydia trachomatis is azithromycin (36, 37).

In our study, in the group treated with doxycycline, after the therapy, 15 % of the patients had a positive smear finding, whereas in the group treated with azithromycin, chlamydia trachomatis after therapy was found in 85 % of the patients.

However, the reasons for the lower efficacy of azithromycin remain unclear. Drug resistance of chlamydia has not been definitively established. Some patients may not have sustained mucosal levels of azithromycin that are sufficient to eradicate chlamydia, and the infection may therefore be driven to a persistent viable state. It is of interest, however, that azithromycin may be more efficacious for treating an upper reproductive tract infection. In a macaque model of pelvic inflammatory disease, azithromycin reduced inflammation and was more effective in eradicating chlamydia from the lower and upper reproductive tract (in 12 of 12 animals) compared with doxycycline (7 of 12 animals) (41). Thus, with the above caveats in mind, it does not seem reasonable to recommend doxycycline over azithromycin as the preferred regimen for chlamydia treatment.

The limitations of this study include a small observed sample of women, particularly with regard to the directly observed administration of the single dose of azithromy-

cin. Because of the finding of very high efficacy for both treatments, we believe that the current therapy empirical recommendation that either drug be used in the treatment of persons with a chlamydia infection seems appropriate and remains valid.

CONCLUSION

Our results confirm that the outcome of infections caused by C. trachomatis depends solely on applied therapy and management, but extensive prospective studies in a female cohort with more included parameters, such as potential age-related, dose-dependent and adherence variability, are necessary to determine and confirm the best choice for treatment of CT cervicitis.

Acknowledgements

This study was conducted with the approval of the Clinical Center Kragujevac Ethics Committee. We thank the staff and patients of The Clinic of Obstetrics and Gynaecology, Clinical Center Kragujevac in Serbia.

Potential conflicts of interest

All authors: No reported conflicts.

REFERENCES

1. Thompson, Sumner E., and A Eugene Washington. Epidemiology of sexually transmitted Chlamydia trachomatis infections. Epidemiologic reviews 5 (1983): 96.
2. Walker, Jennifer, et al. Chlamydia trachomatis incidence and re-infection among young women–behavioural and microbiological characteristics. PloS one 7.5 (2012): e37778.
3. Weinstock, Hillard, Stuart Berman, and Willard Cates. Sexually transmitted diseases among American youth: incidence and prevalence estimates, 2000. Perspectives on sexual and reproductive health 36.1 (2004): 6-10.
4. Stamm, Walter E. Chlamydia trachomatis infections: progress and problems. Journal of Infectious Diseases 179.Supplement 2 (1999): S380-S383.
5. Fenton, Kevin A., et al. Sexual behaviour in Britain: reported sexually transmitted infections and prevalent genital Chlamydia trachomatis infection. The Lancet 358.9296 (2001): 1851-1854.
6. Morré, Servaas A., et al. The natural course of asymptomatic Chlamydia trachomatis infections: 45% clearance and no development of clinical PID after one-year follow-up. International journal of STD & AIDS 13.suppl 1 (2002): 12-18.
7. Cates, Willard, and Judith N. Wasserheit. Genital chlamydial infections: epidemiology and reproductive sequelae. American journal of obstetrics and gynecology 164.6 (1991): 1771-1781.
8. Satterwhite, Catherine Lindsey, et al. Sexually transmitted infections among US women and men: prevalence and incidence estimates, 2008. Sexually transmitted diseases 40.3 (2013): 187-193.
9. de Figueirêdo, Camila Macêdo, et al. Analysis Of Scientific Production On Ophthalmia Case Study Of Newborn And Its Relationship With The Type Of Birth And Preventive Methods: Systematic Review And Meta-Analysis. International Archives of Medicine 9.1 (2016).
10. Ferreira, Glenda Roberta Oliveira Naiff. Soroepidemiologia de Chlamydia trachomatis, Chlamydia pneumoniae e Treponema pallidum nas aldeias indígenas Bakajá, Apyterewa, Xingu e Mrotidjãm, Altamira, Pará, Brasil. (2010).
11. Haggerty, Catherine L., et al. Risk of sequelae after Chlamydia trachomatis genital infection in women. Journal of Infectious diseases 201.Supplement 2 (2010): S134-S155.
12. Jaschek, G., et al. Direct detection of Chlamydia trachomatis in urine specimens from symptomatic and asymptomatic men by using a rapid polymerase chain reaction assay. Journal of clinical microbiology 31.5 (1993): 1209-1212.
13. Morré, S. A., et al. Urogenital Chlamydia trachomatis serovars in men and women with a symptomatic or asymptomatic infection: an association with clinical manifestations?. Journal of Clinical Microbiology 38.6 (2000): 2292-2296.
14. Lee, Helen H., et al. Diagnosis of Chlamydia trachomatis genitourinary infection in women by ligase chain reaction assay of urine. The Lancet 345.8944 (1995): 213-216.
15. Stephens, R. S., et al. Monoclonal antibodies to Chlamydia trachomatis: antibody specificities and antigen characterization. The Journal of Immunology 128.3 (1982): 1083-1089.
16. Ratti, Giulio. Chlamydia trachomatis antigens. U.S. Patent No. 8,114,401. 14 Feb. 2012.
17. Chen, Ding, et al. Characterization of Pgp3, a Chlamydia trachomatis plasmid-encoded immunodominant antigen. Journal of bacteriology 192.22 (2010): 6017-6024.
18. Baud, D., L. Regan, and G. Greub. Comparison of five commercial serological tests for the detection of anti-Chlamydia trachomatis antibodies. European journal of clinical microbiology & infectious diseases 29.6 (2010): 669-675.
19. Stevens, Matthew P., et al. Development and evaluation of an ompA quantitative real-time PCR assay for Chlamydia trachomatis serovar determination. Journal of clinical microbiology 48.6 (2010): 2060-2065.
20. Horner, Paddy J. Azithromycin antimicrobial resistance and genital Chlamydia trachomatis infection: duration of therapy may be the key to improving efficacy. Sexually transmitted infections 88.3 (2012): 154-156.
21. Lanjouw, E., et al. 2010 European guideline for the management of Chlamydia trachomatis infections. International journal of STD & AIDS 21.11 (2010): 729-737.

22. Bhengraj, Apurb Rashmi, et al. Decreased susceptibility to azithromycin and doxycycline in clinical isolates of Chlamydia trachomatis obtained from recurrently infected female patients in India. Chemotherapy 56.5 (2010): 371-377.
23. Pitsouni, Eleni, et al. Single-dose azithromycin versus erythromycin or amoxicillin for Chlamydia trachomatis infection during pregnancy: a meta-analysis of randomised controlled trials. International journal of antimicrobial agents 30.3 (2007): 213-221.
24. Hathorn, Emma, Catherine Opie, and Penny Goold. What is the appropriate treatment for the management of rectal Chlamydia trachomatis in men and women?. Sexually transmitted infections 88.5 (2012): 352-354.
25. Thorpe, E. M., et al. Chlamydial cervicitis and urethritis: single dose treatment compared with doxycycline for seven days in community based practises. Genitourinary medicine 72.2 (1996): 93-97.
26. Schwebke, J. R., et al. Re-evaluating the treatment of nongonococcal urethritis: emphasizing emerging pathogens–a randomized clinical trial. Clinical Infectious Diseases 52.2 (2011): 163-170.
27. Kong, F. Y. S., et al. Azithromycin versus doxycycline for the treatment of genital chlamydia infection–a meta-analysis of randomised controlled trials. Clinical Infectious Diseases (2014): ciu220.
28. Kong, Fabian Yuh Shiong, and Jane Simone Hocking. Treatment challenges for urogenital and anorectal Chlamydia trachomatis. BMC infectious diseases 15.1 (2015): 1.
29. Kissinger, Patricia J., et al. Azithromycin treatment failure for Chlamydia trachomatis among heterosexual men with nongonococcal urethritis. Sexually Transmitted Diseases 43.10 (2016): 599-602.
30. Lau, Chuen-Yen, and Azhar K. Qureshi. Azithromycin versus doxycycline for genital chlamydial infections: a meta-analysis of randomized clinical trials. Sexually transmitted diseases 29.9 (2002): 497-502.
31. Rustomjee, Roxana, et al. A randomized controlled trial of azithromycin versus doxycycline/ciprofloxacin for the syndromic management of sexually transmitted infections in a resource-poor setting. Journal of Antimicrobial Chemotherapy 49.5 (2002): 875-878.
32. Gambini, Daniele, et al. Mycoplasma genitaliumin Males With Nongonococcal Urethritis: Prevalence and Clinical Efficacy of Eradication. Sexually transmitted diseases 27.4 (2000): 226-229.
33. Ibrahim Salah Eldin. Evaluation Of Azithromycin In Treatment Of Avulgaris. Diss. UOFK, 2015.
34. Manhart, Lisa E., et al. Efficacy of antimicrobial therapy for Mycoplasma genitalium infections. Clinical Infectious Diseases 61.suppl 8 (2015): S802-S817.
35. Perletti, Gianpaolo, et al. Antimicrobial therapy for chronic bacterial prostatitis. The Cochrane Library (2013).
36. Haddix, Anne C., Susan D. Hillis, and William J. Kassler. The cost effectiveness of azithromycin for Chlamydia trachomatis infections in women. Sexually Transmitted Diseases 22.5 (1995): 274-280.
37. Genc, Mehmet, and Per-Anders Mardh. A cost-effectiveness analysis of screening and treatment for Chlamydia trachomatis infection in asymptomatic women. Annals of Internal Medicine 124.1_Part_1 (1996): 1-7.

PREVALENCE OF PROLONGED QTC INTERVAL IN PATIENTS TAKING PSYCHOPHARMACS

Zoran Jovanovic[1], Vesela Radonjic[2], Ratomir Jelic[2], Narcisa Petrovic-Subic[3], Ivan Soldatovic[1], Vera Terzic[1], Sladjan Stojilkovic[4], Dusan Djuric[2]
[1] General hospital Sabac, Department of Psychiatry, Republic of Serbia
[2] Department of pharmacy, Faculty of medical sciences, Kragujevac, Republic of Serbia
[3] Hemofarm A.D. Vršac, Serbia,
[4] Society of external and internal export Medicom, Sabac, Republic of Serbia

ZASTUPLJENOST PRODUŽENOG QTC INTERVALA KOD PACIJENATA KOJI UZIMAJU PSIHOFARMAKE

Zoran Jovanović[1], Vesela Radonjić[2], Ratomir Jelić[2], Narcisa Petrović-Šubić[3], Ivan Soldatović[1], Vera Terzić[1], Sladjan Stojilković[4], Dušan Đurić[2]
[1] Opšta bolnica Šabac, Služba psihijatrije, Republika Srbija
[2] Katedra za farmaciju, Fakultet medicinskih nauka, Kragujevac, Republika Srbija
[3] Hemofarm A.D, Republika Srbija
[4] Društvo za spoljnu i unutrašnju trgovinu Medicom d.o.o. Šabac, Republika Srbija

Received / Primljen: 15. 08. 2016. Accepted / Prihvaćen: 05. 12. 2016.

ABSTRACT

Apart from providing knowledge on the beneficial effects of drugs, practical psychopharmacotherapy also includes drug profiles of adverse effects, especially when medical comorbidity is present. The mechanism of action of many psychotropic drugs, mainly antipsychotics and antidepressants, is associated with prolongation of the QT interval and the occurrence of arrhythmias, specifically Torsade de pointes (TdP), which can be lethal. The aim of this pilot study was to confirm the prevalence of prolonged QTc interval in a sample of psychiatric patients taking psychopharmacs.

The present study included 41 patients who were already on psychopharmacs. The average value of the QTc interval in the observed sample was 413.8±23.3 ms. The most frequent psychopharmacotherapy was the combination of typical and atypical antipsychotics (24.4%), followed by monotherapy with antipsychotics (22%) and combined antidepressant and atypical antipsychotic therapy (22%). The average value of the QTc interval for male patients was 412.1±25.2 ms, whereas for female patients, it was 416.6±20.4 ms. No difference between sexes was confirmed (p=0.555). The correlation between the QTc interval and age of patients was positive but not statistically significant (p=0.072). The highest average (419.3±31.6 ms) and highest maximum (479 ms) values of the QTc interval were noted for patients undergoing combined therapy of antidepressants and atypical antipsychotics. Prolonged values of the QTc interval were observed for seven males and one female, and no patients exhibited pathological values.

This study confirmed previous research that found that prolongation of the QTc interval exists in patients in sample groups who take psychopharmacs, but not up to critical values.

Keywords: *psychopharmacs, antidepressants, antipsychotics, prolonged QTc interval, Torsade de pointes*

SAŽETAK

Racionalna psihofarmakoterapija podrazumeva osim dobrog poznavanja blagotvornih efekata lekova i njihov profil neželjenih dejstava, posebno kada postoji somatski komorbiditet. Mnogi psihotropni lekovi, prvenstveno antipsihotici i antidepresivi, povezani su sa produženjem QT intervala i nastankom aritmija tipa Torsade de pointes (TdP), što se može završiti letalno. Cilj ove pilot studije bio je da, kod posmatrane grupe psihijatrijskih pacijenata koji uzimaju psihofarmake, utvrdimo zastupljenost produženog QTc inervala.

U ispitivanje je uključen 41 bolesnik, koji već koriste psihofarmake u terapiji. Naše istraživanje je pokazalo da je prosečna vrednost QTc intervala u posmatranom uzorku iznosila 413,8±23,3 ms. Najčešće je primenjivana kombinacija tipičnog i atipičnog antipsihotika (24,4%), sledi monoterapija antipsihotikom i kombinovana terapija antidepresiv-atipični antipsihotik sa po 22%. Prosečna vrednost QTc intervala kod muškaraca iznosila je 412,1±25,2 ms a kod žena 416,6±20,4ms i nije utvrđena razlika između polova (p=0,555). Korelacija QTc intervala i starosti pacijenata bila je pozitivna ali nije statistički značajna (p=0,072). Najviše prosečne (419,3±31,6ms) kao i maksimalne (479ms) vrednosti QTc intervala uočene su kod pacijenata na kombinovanoj terapiji antidepresiv i atipični antipsihotik. Produžene vrednosti QTc intervala registrovane su kod sedam bolesnika i kod jedne bolesnice dok patoloških vrednosti u posmatranom uzorku nije bilo.

Ova studija potvrdila je prethodna saznanja da prolongacija QTc intervala postoji kod pacijenata u našem uzorku koji uzimaju psihofarmake, ali ne i do kritičnih vrednosti.

Ključne reči: *psihofarmaci, antidepresivi, antipsihotici, produžen QTc interval, Torsade de pointes*

sciendo

UDK: 615.214.06; 616.12-073.97 / Ser J Exp Clin Res 2018; 19 (2): 159-166
DOI: 10.1515/SJECR-2016-0096

Corresponding author: Zoran Jovanovic,
General hospital Sabac, Department of Psychiatry, Republic of Serbia,
e-mail: zoranjov73@gmail.com

INTRODUCTION

The complexity of mental disorders is apparent not only in their primary symptoms but also in the adverse effects of available pharmacotherapies. Oftentimes, the adverse symptoms are not noticed in a timely manner due to the patients' altered psychological functioning, negligence of their own health and ascribing iatrogenic symptoms to a disease itself (1-3). Although new psychopharmacs generally have fewer adverse effects, we are still not satisfied with the level of efficacy and safety (4). Furthermore, psychiatric patients are likely to abuse psychoactive substances and experience drug overdose, which makes the clinical picture more complex and significantly changes the bioavailability of psychotropic drugs (5, 6). Given the above complications, psychopharmacs are a cause of many adverse events and have the potential for interactions with drugs of other categories (4).

Although rare, iatrogenic cardiovascular disorders are amongst the most serious adverse events and mainly include heart rhythm disorders and sudden cardiac death (7, 8). Many psychopharmacs, especially antipsychotics and antidepressants, are associated with prolongation of the QT interval and the occurrence of arrhythmia, specifically the type *Torsade de pointes* (TdP), which can be lethal (9, 10). The QT interval on an electrocardiogram represents the time interval from the beginning of the Q wave to the end of the T wave, i.e., it corresponds to the start of activation (depolarization) and the end of recovery (repolarization) of the ventricles. Its physiological values are variable due to the influence of heart rate, age and sex, thus, as a more precise electrophysiological value, the corrected value QTc interval is used (11). Normal values of QTc interval for males reach up to 430 ms, borderline values range from 431 ms to 450 ms, and pathological values are >450 ms; whereas for females, normal values reach up to 450 ms, borderline values range from 451 ms to 470 mg and pathological values are >470 ms (12). Prolongation of the QTc value above 500 ms for both sexes is not always accompanied by arrhythmia, but it significantly increases the risk of TdP occurrence, in other words, sudden cardiac death (13).

The potential for prolongation of the QT interval by psychopharmacs was first noticed with phenothiazines, mostly with thioridazine (approximately 36 ms), and therefore, it was generally withdrawn from the market. However, thioridazine is still administered in some countries under specific circumstances (14). Subsequently, numerous studies confirmed that many other antipsychotics can variably prolong the QT interval, such as pimozide, chlorpromazine, haloperidol and droperidol; however, more recent drugs, such as sertindole, quetiapine, ziprasidone, risperidone, etc., can also prolong the QT interval (15, 16). In addition, the same effect was noticed with methadone (17), lithium and different antidepressants, including tri- and tetra-cyclic antidepressants and SSRI/SNRI [(es)citalopram, fluoxetine, venlafaxine, etc.] (16). Additional factors that can increase the risk of prolonged QT interval include inborn prolongation of the QT interval, comorbid cardiac and other medical diseases, anorexia, electrolyte imbalance, simultaneous intake of other drugs that prolong QT interval or interact with psychopharmacs, age older than 65 years, female sex and positive familial anamnesis of sudden cardiac death (18, 19). For this reason, testing is required on the pro-arrhythmogenic potential of drugs, i.e., their the influence on QT/QTc interval, during pre-clinical and clinical trials of these drugs (20, 21).

The aim of this pilot study was to confirm the prevalence of prolonged QTc interval in a group of psychiatric patients who take psychopharmacs.

PATIENTS AND METHODOLOGY

This cross-sectional study used data from 41 hospitalized patients in the psychiatric ward of the General Hospital in Sabac after obtaining informed consent. The sample involved patients of both sexes, aged 24 to 80 years old, who were already taking psychopharmacs. The patients were stratified into five groups depending on whether they took antidepressant monotherapy, atypical/typical antipsychotic therapy, combined therapy with antidepressant/antipsychotic or substitutional methadone therapy in the last seven days. The observed groups underwent additional therapy of benzodiazepine and/or psychostabilizers. All the patients underwent a standard 12-lead electrocardiogram (ECG). Only ECGs of technical quality that did not register disorders of conduction of the His bundle (block/chemiblock), ST segments or T waves were considered. The exclusion criteria for this study were patients who took neuropsychiatric drugs, which, according to the relevant references, can extend the QTc interval (22, 23). The basic psychiatric diagnoses of the participants were given in accordance with the International Classification of Disease – 10th revision (ICD-10).

The length of the QT interval depends on the patient's sex and heart rate. The value of the QTc interval is expressed in milliseconds (ms) and the obtained results, according to the valid international criteria (12), were classified into six intervals: normal values for men up to 430 ms, normal values for women up to 450 ms, borderline values for men up to 431-450 ms, borderline values for women up to 451-470 ms, pathological values for men >450 ms and pathological values for women >470 ms. For all patients, the corrected QT interval (QTc) was calculated according to Bazett's formula, in which the value of the QT interval (QT) is divided by the square root of the length between two consecutive R waves ($QTc=QT/\sqrt{RR}$). In order to determine the end of the T wave in unclear ECGs, the QT interval was calculated using the tangent method in cycles where the T wave was best defined.

The results are presented as arithmetic means +/- standard deviations or as a number (percentage). The t-test and ANOVA were used to test the significance of the differences in numerical observations, whereas the Chi square test

Table 1 The comparison of the QTc interval by sex of patients taking psychopharmacs

Sex	Arithmetic mean QTc	SD	Median	Minimum	Maximum
Male	412.1	25.2	413.0	360	462
Female	416.6	20.4	408.5	389	479

was used to examine the significance of differences among groups concerning nominal observations. Pearson correlation analysis was used to examine the correlation between two numerical variables. All the results were processed using SPSS 20.0 (IBM corporation) software package.

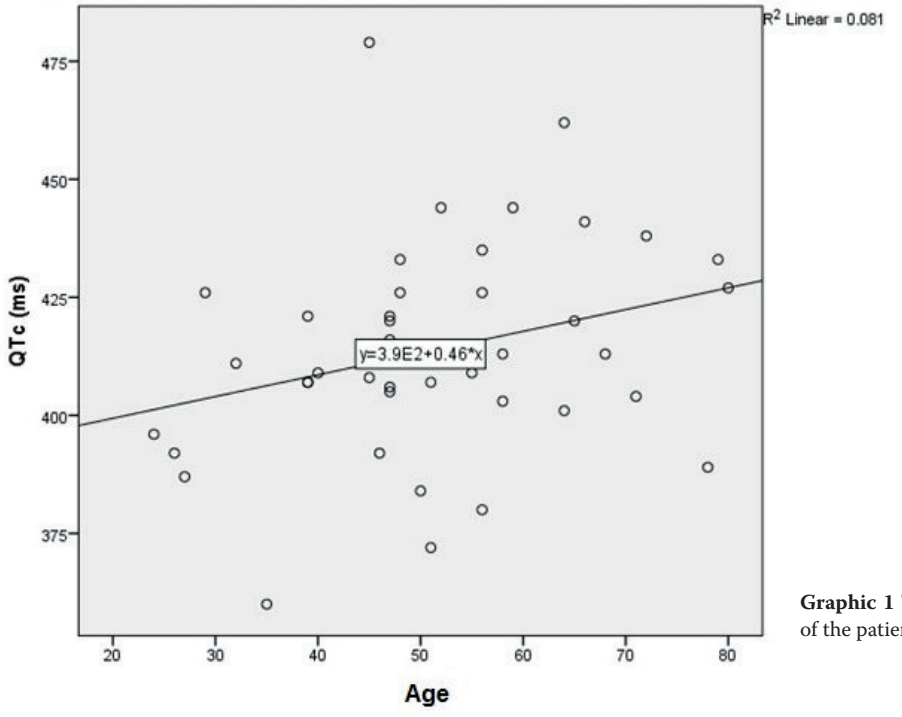
RESULTS

The observed group included 41 patients, consisting of 25 males (61%) and 16 (39%) females, with an average age of 51.3±14.4 years. The average age of male patients was 50.2 years, whereas the average age of female patients was 53.2 years; there was no statically significant difference in age between sexes (p=0.509). The average value of the QTc interval in the observed sample was 413.8±23.3 ms with a range of 360 ms to 479 ms. The most common diagnoses were various schizophrenic disorders in 48.7% of patients [i.e., schizophrenia (F20) 36.6%, schizoaffective disorder (F25) 7.3%, brief psychotic disorder (F23) 2.4% and unspecified psychosis (F29) 2.4%]. Other diagnoses were affective disorders in 36.6% of patients [i.e., recurrent depressive disorder (F33) 24.4%, single episode of depressive disorder (F32) 9.6%, and bipolar affective disorder (F31) 2.4%], chronic psychoorganic syndrome (F06) in 9.8% of patients, and dementia (F03) and opioid-related disorders (F11) in 2.4% of patients. Regarding pharmacotherapy, most patients were taking a combination of typical and atypical antipsychotics (24.4.%), followed by monotherapy of antipsychotic (22%), combined therapy of antidepressant – atypical antipsychotic (22%), monotherapy of antidepressant (17.1%), typical antipsychotic (12.2%) and substitutional methadone therapy (2.4%).

In **Table 1**, the values of the QTc interval by patient sex are presented. The average value for males is 412.1±25.2 ms, whereas for females the average value is 416.6±20.4 ms. There was no statistically significant difference in QTc interval between sexes (t=0.596; p=0.555).

The correlation between QTc interval and age was weak to moderately positive but not statistically significant (r=0.284; p=0.072) (**Graphic 1**). The highest independent value of 479 ms for the QTc interval was recorded in a 46-year-old female patient.

The relationship between pharmacotherapy and the values of the QTc intervals were analysed. The average QTc values by medications are shown in **Table 2**. The highest average QTc interval (419.3±31.6 ms) and the overall maximal value (479 ms) were noted in the group of patients undergoing combined therapy of an antidepressant and an atypical antipsychotic. When excluding the one patient taking methadone therapy, there was no statistically significant difference between the duration of the QTc interval by administered therapy, regardless of whether it was monotherapy or a combination of psychopharmacs (F=0.522; p=0.720).



Graphic 1 The correlation between QTc interval and age of the patients taking psychopharmacs

Table 2 The comparison of the values of the QTc interval according to pharmacotherapeutic group

Th	Arithmetic mean QTc	SD	Median	Minimum	Maximum
Antidepressant	405.0	20.1	401.0	380	433
Typical antipsychotic	406.6	21.2	404.0	387	441
Atypical antipsychotic	416.0	10.3	413.0	407	435
Antidepressant + atypical antipsychotic	419.3	31.6	420.0	360	479
Typical antipsychotic +atypical antipsychotic	417.0	28.7	414.0	372	462
Methadone	411.0	.	411.0	411	411

Box-plot **Diagram 1** shows the distribution of the QTc interval depending on the type of psychopharmacotherapy.

The measured values of the QTc interval were, in accordance with the valid international criteria (12), classified in six intervals: normal values for men up to 430 ms, normal values for women up to 450 ms, borderline values for men 431-450 ms, borderline values for women 451-470 ms, pathological values for men >450 ms and pathological values for women>470 ms. Prolonged values were noted for 7 male patients and one female patient, but there were no patients with pathological values. There was no statistically significant difference in QTc interval categories between sexes (p=0.120) (**Table 3**).

Table 3 QTc interval categories by sex of the patients taking psychopharmacs

Values QTc				
Sex		Normal	Higher	Total
Male	N	18	7	25
	%	72.00%	28.00%	100.00%
Female	N	15	1	16
	%	93.80%	6.30%	100.00%

In addition, the correlation between the abovementioned QTc categories and administered psychotropic drugs was analysed. According to the statistical parameters, the sample was too small for such a large number of categories. When one patient undergoing methadone therapy was excluded, it was confirmed that there was no statistically significant difference between the observed QTc categories and the administered psychotropic drugs (p=0.310). The exact test was used for this analysis. On an individual basis, in one 46-year-old female patient who was administered clomipramine 150 mg in combination with atypical antipsychotic quetiapine 600 mg per day, the QTc interval was 479 ms. For the seven male patients with QT prolongation, a maximum QTc interval of 462 ms was recorded, and all of them were administered different drug categories. Four of these patients took combined therapy chlorpromazine in dosages from 50 to 150 mg. The relationship between pharmacotherapy and QTc interval values is shown in **Table 4**.

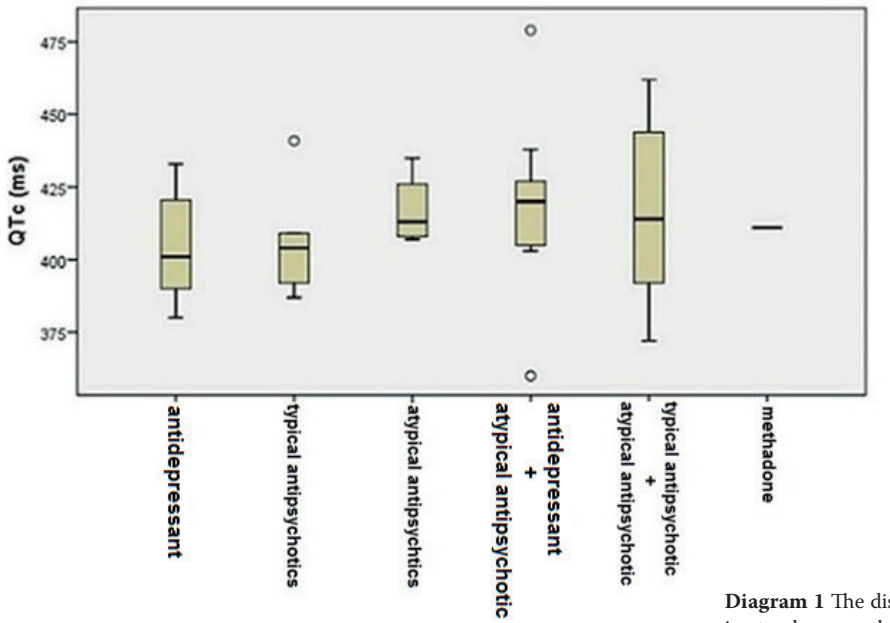
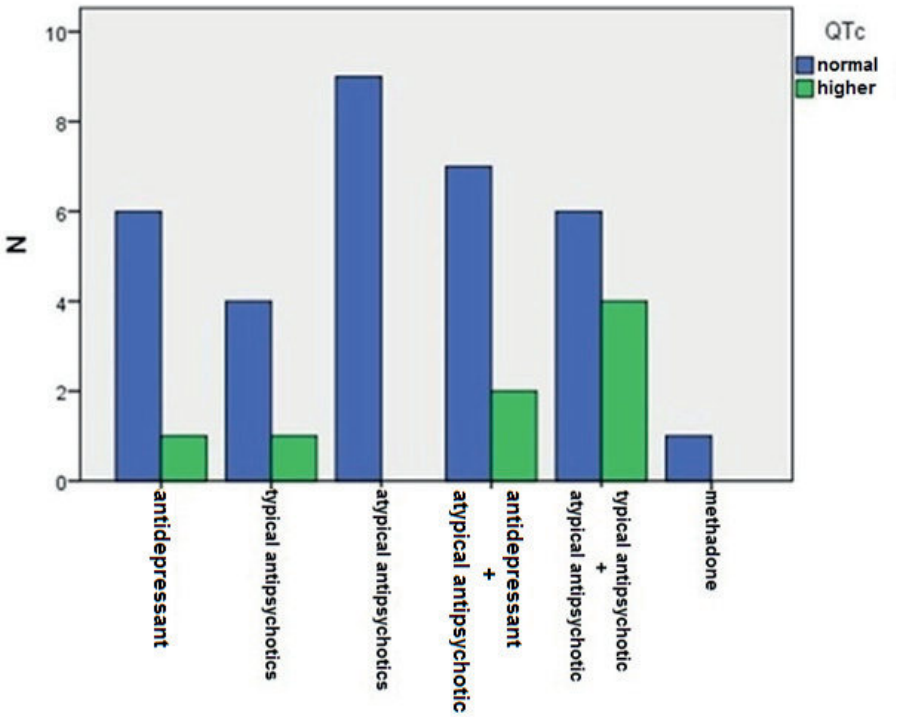


Diagram 1 The distribution of values of the QTc interval according to pharmacotherapeutic group

Diagram 2 QTc intervals according to pharmacotherapeutic groups



DISCUSSION

Diagram 2 shows that the prolonged value of the QTc interval was mostly present in patients taking combined therapy of typical and atypical antipsychotics, followed by combined therapy of antidepressant and atypical antipsychotic. However, QTc prolongation was less prominent in monotherapy using an antidepressant and an atypical antipsychotic.

In this pilot study, participants of both sexes were included equally. In addition, the geriatric population with mental problems was also present, since, in our society, they are often hospitalized at psychiatric wards, at least at the secondary level. The most common were patients who suffer from disorders of the schizophrenia spectrum (F20-F29), followed by affective disorders (F31-F33), chronic

Table 4 The comparison QTc interval categories according to the pharmacotherapeutic groups

QTc categories				
Th		Normal	Higher	Total
Antidepressant	N	6	1	7
	%	85.70%	14.30%	100.00%
Atypical antipsychotics	N	4	1	5
	%	80.00%	20.00%	100.00%
Atypical antipsychotics	N	9	0	9
	%	100.00%	0.00%	100.00%
Antidepressant+atypical antipsychotic	N	7	2	9
	%	77.80%	22.20%	100.00%
Typical antipsychotic +atypical antipsychotic	N	6	4	10
	%	60.00%	40.00%	100.00%
Methadone	N	1	0	1
	%	100.00%	0.00%	100.00%

psychoorganic syndrome (F06) and dementia (F03), which required hospital treatment due to the complexity of the clinical picture.

Our patients did not show prolongation of QTc interval above the critical value of 500 ms, which, according to all current recommendations, would be a reason to change pharmacotherapy (10). Nevertheless, there is no data on the values of the QTc interval in psychiatric patients before psychopharmacs were introduced. Therefore, it was not possible to calculate whether and to what extent there was prolongation of QTc interval, especially more than 60 ms, in comparison to a baseline value. Although there were no statistically significant differences, the average value of the QTc interval for male patients was lower than the average values for female patients (412.1±25.23 ms *vs*. 416.6±20.4 ms), which is in accordance with previous research. The shorter QTc for male patients compared to female patients can be explained by a quicker start of repolarization in male patients. This gender-specific difference decreases with age and is probably caused by the effects of testosterone on calcium current (25). Contrary to the abovementioned findings, in our sample, prolongation of the QTc interval was noted for seven male patients and one female patient. Comparing the age and the QTc values, a weak to average correlation between QTc interval and age of the patients was confirmed. Although a significant difference was not noted for ventricular repolarization by pharmacotherapeutic group, the highest value of QTc interval (419.3±31.6 ms) was observed in patients who were taking a combination of antidepressants and atypical antipsychotics; slightly lower values of QTc intervals were observed for patients who were on monotherapy of an antidepressant or an antipsychotic. These results are equal to other published research (26), though many authors confirm a significantly higher risk of prolongation of QTc interval with psychopharmacs polytherapy (27). Although a statistically significant difference was not confirmed, an analysis of the relationship between administered monotherapy or combination of drugs and QTc categories led to almost identical results. In the 40% of patients taking a combination of typical and atypical antipsychotics, prolongation of the QTc interval was confirmed, as well as in the 22.2% of patients on a combination of an antidepressant and an atypical antipsychotic. This finding can be explained by the additive effect of these drugs on ventricular repolarization. To emphasize, the combinations of drugs with high potential for prolongation of QTc values should be avoided when possible, especially in patients with other known risk factors (18,19). Relevant research shows that the greatest negative effect is observed with phenothiazines (thioridazine 36 ms, pimozide 19 ms), followed by sertindole 30 ms, ziprasidone 15.9 ms, haloperidol 7.1 ms, quetiapine 5.7 ms and risperidone 3.6 ms (28,29). With regard to clozapine, the results of this study are conflicting, but its cardiotoxic potential is unquestionable (30). Among antidepressants, mainly the adverse effects of tricyclic and tetracyclic were recorded (clomipramine, maprotiline, nortriptyline, amitriptyline), as well as the dosage-additive potential of citalopram (18.5 ms in dosages of 60 mg/day) and escitalopram (10.7 ms in dosages of 30 mg/day) (31). The study also suggests clinically significant effect of recent drugs, such as venlafaxine, especially in dosages more than 300 mg per day, and bupropion. Furthermore, research thus far has also confirmed the adverse influence of methadone substitutional therapy on QTc values (32).

STUDY LIMITATIONS

The main drawback of the study was that the number of patients in the present study was rather small compared to the data from the available literature. Other limitations include the lack of more precise exclusion criteria according to cardiology and general practice guidelines, laboratory analyses and baseline values of the QTc interval before administration of psychopharmacs. With more strict exclusion criteria, it would be possible to avoid potential influence of other factors on repolarization of ventricles, not only those accounted for by physical examination in psychiatric practice, anamnesis of somatic diseases and patients not taking neuropsychiatric drugs. In addition, there are also deviations of basic values during drug administration. The question of a valid determination of QTc interval can be raised concerning this and concerning broader research that has been carried out so far. Namely, the international research carried out by Viskin and associates (33) showed that most medical doctors (25%), including many cardiologists, cannot precisely calculate QTc and cannot accurately identify prolonged QT. In addition, agitation of patients can influence electrolyte balance, i.e., cause hypokalemia, which would additionally change ventricular repolarization (34). Circadian changes and even consumption of certain foods, beverages and supplements can also influence values of QTc (35,36). Additionally, the complexity of the problem is caused by various recommendations concerning the time of recording the ECG, where most experts recommend recordings 12 hours after drug administration or 30 to 60 minutes after its peak blood concentration is achieved (37,38).

CONCLUSION

Prolongation of the QTc interval caused by psychopharmacs, mainly antipsychotics and antidepressants, is a relatively rare but severe event that can be lethal. Since administration of psychotropic drugs in daily practice is inevitable, an integrative approach to patients is necessary to avoid this kind of adverse event and to react effectively in such cases This strategy means knowing the mechanisms of action of a drug and methods of individual assessment of all the mentioned risks and, if necessary, the ability to interpret electrolytes and ECGs. The balance among the risks and positive effects of psychopharmacs is a great

challenge, especially in our society where a psychiatrist is very often "a general practitioner" who also takes care of the general health of his/her patients.

Taking into consideration all the abovementioned limitations, this study showed that prolongation of the QTc interval exists in patients in our society who take psychopharmacs, but it does not lead to critical, i.e., torsadogenic, values.

To decrease the incidence of psychotropic-related QT prolongation, it is necessary to conduct further and broader clinical and pre-clinical research.

REFERENCES

1. Kang SH, Lee JI. Metabolic disturbances independent of body mass in patients with schizophrenia taking atypical antipsychotics. Psychiatry Investig. 2015; 12(2):242-8.
2. Rouillon F, Van Ganse E, Vekhoff P, Arnaud R, Depret-Bixio L, Dillenschneider A. Vigilance level for cardiovascular risk factors in schizophrenic patients. Encephale. 2015;41(1):70-7.
3. Cradock-O'Leary J, Young AS, Yano EM, Wang M, Lee ML. Use of general medical services by VA patients with psychiatric disorders. Psychiatr Serv. 2002;53(7):874-8.
4. Jones ME, Campbell G, Patel D, Brunner E, Shatapathy CC, Murray-Thomas T, van Staa TP, Motsko S. Risk of Mortality (including Sudden Cardiac Death) and Major Cardiovascular Events in Users of Olanzapine and Other Antipsychotics: A Study with the General Practice Research Database. Cardiovasc Psychiatry Neurol. 2013;2013:647476.
5. Tolliver BK, Anton RF. Assessment and treatment of mood disorders in the context of substance abuse. Dialogues Clin Neurosci. 2015;17(2):181-90.
6. Mowry JB, Spyker DA, Cantilena LR Jr, McMillan N, Ford M.2013 Annual Report of the American Association of Poison Control Centers' National Poison Data System: 31th Annual Report. Clin Toxicol. 2014;52:1032–283.
7. Weeke P, Jensen A, Folke F, Gislason GH, Olesen JB, Fosbøl EL, Wissenberg M, Lippert FK, Christensen EF, Nielsen SL, Holm E, Kanters JK, Poulsen HE, Køber L, Torp-Pedersen C. Antipsychotics and associated risk of out-of-hospital cardiac arrest. Clin Pharmacol Ther. 2014;96(4):490-7.
8. Nezafati MH, Vojdanparast M, Nezafati P. Antidepressants and cardiovascular adverse events: A narrative review. ARYA Atheroscler. 2015;11(5):295-304.
9. Zemrak WR, George A. Kenna GA. Association of Antipsychotic and Antidepressant Drugs With Q-T interval Prolongation. Am J Health Syst Pharm. 2008;65(11):1029-1038.
10. Hasnain M, Vieweg WV. QTc interval prolongation and torsade de pointes associated with second-generation antipsychotics and antidepressants: a comprehensive review. CNS Drugs. 2014;28(10):887-920.
11. Postema PG, Wilde AA. The Measurement of the QT Interval. Current Cardiology Reviews. 2014;10(3):287-294.
12. Committee for Proprietary Medicinal Products. The assessment of the potential for QT interval prolongation by non-cardiovascular medicinal products, Committee for Proprietary Medicinal Products, London (1997).
13. De Bruin ML, Langendijk PN, Koopmans RP, Wilde AA, Leufkens HG, Hoes AW. In-hospital cardiac arrest is associated with use of non-antiarrhythmic QTc-prolonging drugs. Br J Clin Pharmacol. 2007;63:216–223.
14. Huffman JC, Stern TA. QTc Prolongation and the Use of Antipsychotics: A Case Discussion. Prim Care Companion J Clin Psychiatry. 2003;5(6):278-281.
15. Vieweg WV. New Generation Antipsychotic Drugs and QTc Interval Prolongation.Prim Care Companion J Clin Psychiatry. 2003;5(5):205-215.
16. Girardin FR, Gex-Fabry M, Berney P, Shah D, Gaspoz JM, Dayer P. Drug-induced long QT in adult psychiatric inpatients: the 5-year cross-sectional ECG Screening Outcome in Psychiatry study. Am J Psychiatry. 2013;170(12):1468-76.
17. Wenzel-Seifert K, Wittmann M, Haen E. QTc Prolongation by Psychotropic Drugs and the Risk of Torsade de Pointes. Dtsch Arztebl Int. 2011; 108(41): 687–693.
18. Alinejad S, Kazemi T, Zamani N, Hoffman RS, Mehrpour O. A systematic review of the cardiotoxicity of methadone. EXCLI J. 2015;14:577-600.
19. Danielsson B, Collin J, Jonasdottir Bergman G, Borg N, Salmi P, Fastbom J. Antidepressants and antipsychotics classified with torsades de pointes arrhythmia risk and mortality in older adults - a Swedish nationwide study. Br J Clin Pharmacol. 2016;81(4):773-83.
20. Drew BJ, Ackerman MJ, Funk M, et al. Prevention of Torsade de Pointes in Hospital Settings: A Scientific Statement From the American Heart Association and the American College of Cardiology FoundationEndorsed by the American Association of Critical-Care Nurses and the International Society for Computerized Electrocardiology. Circulation. 2010;121(8):1047-1060.
21. Anonymous (2005b). International Conference on Harmonisation; guidance on S7b Nonclinical evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals; availability. Notice. Fed Regist 70:61133–61134.
22. Anonymous (2005a). International Conference on Harmonisation; guidance on E14 Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs; availability. Notice. Fed Regist 70: 61134–61135.
23. CredibleMeds® QTDrugs Lists [cited 05 August 2014]. Available: https://www.crediblemeds.org. Accessed 5 June 2016.
24. Schächtele S, Tümena T, Gaßmann KG, Fromm MF, Maas R. Co-Prescription of QT-Interval Prolonging Drugs: An Analysis in a Large Cohort of Geriatric Patients. PLoS One. 2016;11(5):e0155649.

25. Vicente J, Johannesen L, Galeotti L, Strauss DG. Mechanisms of sex and age differences in ventricular repolarization in humans. Am Heart J. 2014;168(5):749-56).
26. Sumić JČ, Barić V, Bilić P, Herceg M, Sisek-Šprem M, Jukić V. QTc and psychopharmacs: are there any differences between monotherapy and polytherapy. Annals of General Psychiatry. 2007;6:13.
27. Sala M, Vicentini A, Brambilla P et al. QT interval prolongation related to psycho-active drug treatment: a comparison of monotherapy versus polytherapy. Ann Gen Psychiatry. 2005; 4:1.
28. Taylor DM. Antipsychotics and QT prolongation. Acta Psychiatr Scand. 2003;107(2):85-95.
29. Harrigan EP, Miceli JJ, Anziano R et al. A randomized evaluation of six antipsychotic agents on QTc, in the absence and presence of metabolic inhibition. J Clin Psychopharmacol. 2004; 24:62-9.
30. Curto M, Girardi N, Lionetto L, Ciavarella GM, Ferracuti S, Baldessarini RJ. Systematic Review of Clozapine Cardiotoxicity. Curr Psychiatry Rep. 2016;18(7):68.
31. Lam RW. Psychopharmacology for the Clinician. J Psychiatry Neurosci. 2013; 38(2): E5–E6.
32. Stringer J, Welsh Ch, Tommasello A. Methadone-associated Q-T Interval Prolongation and Torsades de Pointes. Am J Health Syst Pharm. 2009;66(9):825-833.
33. Viskin S, Rosovski U, Sands AJ, Chen E, Kistler PM, Kalman JM, Rodriguez Chavez L, Iturralde Torres P, Cruz FFE, Centurion OA, Fujiki A, Maury P, Chen X, Krahn AD, Roithinger F, Zhang L, Vincent GM, Zeltser D: Inaccurate electrocardiographic interpretation of long QT: The majority of physicians cannot recognize a long QT when they see one. Heart Rhythm 2005;2: 569-574.
34. M.Vega, C. Riaza, A. Chinchilla, I. Gobernado. HYPOKALEMIA, PSYCHOMOTOR AGITATION AND PROLONGING THE QTC. European Psychiatry 2011.
35. Molnar J, Zhang F, Weiss J, Ehlert FA, Rosenthal JE. Diurnal pattern of QTc interval: how long is prolonged? Possible relation to circadian triggers of cardiovascular events. J Am Coll Cardiol. 1996;27(1):76–83.
36. Zitron E, Scholz E, Owen RW, Lück S, Kiesecker C, Thomas D, et al. QTc prolongation by grapefruit juice and its potential pharmacological basis: HERG channel blockade by flavonoids. Circulation. 2005;111:835–838.
37. Goldenberg I, Moss AJ, Zareba W. QT Interval: How to Measure It and What Is "Normal". J Cardiovasc Electrophysiol. 2006;17(3):333-6.
38. Ames D, Camm J, Cook P, Falkai P, Gury C, Hurley R, Johnson G, Piepho R, Vieweg V; Cardiac Safety in Schizophrenia Group. [Minimizing the risks associated with QTc prolongation in people with schizophrenia. A consensus statement by the Cardiac Safety in Schizophrenia Group]. Encephale. 2002; 28(6 Pt 1):552-62.

TEST ANXIETY IN PRE-EXAM PERIOD AND SUCCESS OF NURSING STUDENTS

Goran Stojanovic[1], Milica Vasiljevic-Blagojevic[1], Bratislav Stankovic[1], Negra Terzic[1], Dragana Terzic-Markovic[1], Dusan Stojanovic[2]
[1]High Medical College of professional Studies in Belgrade, Serbia
[2]Dea medica, Belgrade, Serbia

ISPITNA ANKSIOZNOST U PREDISPITNOM PERIODU I USPEH STUDENATA SESTRINSTVA

Goran Stojanović[1], Milica Vasiljević-Blagojević[1], Bratislav Stanković[1], Negra Terzić[1], Dragana Terzić-Marković[1], Dušan Stojanović[2]
[1]Visoka zdravstvena škola strukovnih studija u Beogradu, Beograd, Srbija
[2]Dea medica, Beograd, Srbija

Received / Primljen: 25. 10. 2016. Accepted / Prihvaćen: 05. 01. 2017.

ABSTRACT

The aim of this study was to examine the presence of pre-exam anxiety in nursing students and establish relationship of pre-exam anxiety intensity in relation to the category variables (gender, age, place of birth, current place of residence, with whom they live, whether they are employed, the average family income, year of study), objective success (i.e., pass rate at exams, the average score at the end of year, possible renewal of the year of study) and the subjective perception of academic success (i.e., self-satisfaction as a student as well as the importance of ratings obtained at the exam). The sample was random and consisted of the students of High Medical College of Professional Studies in Belgrade at the Department of vocational nurse. 209 students were tested, evenly distributed on the second and third year of study. Pre-examination anxiety among students was examined using a questionnaire The Test Anxiety Inventory – TAI (Test Anxiety Inventory), which includes subscales: Test Anxiety Inventory-Total (TAI-T), Test Anxiety Inventory-Worry (TAI-W) and Test Anxiety Inventory-Emotionality (TAI-E). From the obtained results we can conclude that nursing students showed a statistically significant pre-exam anxiety on all subscales. Pre-exam anxiety symptoms compared to candidates' sex showed statistically significant differences in all scores, and average values are always higher in female students compared to male. There is also a statistically significant difference between students of the second year in relation to the third year students. It can be concluded that there is a significant number of nursing students with the pre-exam anxiety problems that need professional help and support in the form of expanding and strengthening personal competencies.

Keywords: *students, study nursing, pre-exam anxiety, stress, exams*

SAŽETAK

Cilj istraživanja je bio da se ispita prisustvo predispitne anksioznosti kod studenata sestrinstva i utvrdi povezanost intenziteta predispitne anksioznosti u odnosu na kategorijalne varijable (pol, uzrast, mesto rođenja, trenutno mesto stanovanja, sa kim žive, da li su zaposleni, prosečna primanja porodice, godinu studija), objektivni uspeh (prolaznost na ispitima, prosečna ocena na kraju godine, moguće obnavljanje godine studija), i subjektivnu percepciju akademskog uspeha (zadovoljstvo sobom kao studentom kao i važnosti dobijene ocene na ispitu). Uzorak je slučajan i sačinjavali su ga studenti Visoke zdravstvene škole strukovnih studija u Beogradu na studijskom Odseku strukovna medicinska sestra. Testirano je 209 studenata, ravnomerno raspoređenih na drugu i treću godinu studija. Predispitna aksioznost među studentima je ispitivana pomoću upitnika za merenje anksioznosti Instrument za ispitivanje aksioznosti eng. Test Anxiety Inventory (TAI), koji sadrži nekoliko subskala: Test Anxiety Inventory-Total (TAI-T), Test Anxiety Inventory-Worry (TAI-W) i Test Anxiety Inventory-Emotionality (TAI-E). Iz dobijenih rezultata možemo zaključiti da studenti sestrinstva pokazuju statistički značajnu predispitnu anksioznost na svim subskalama. Simptomi predispitne anksioznosti u odnosu na pol kandidata pokazali su statistički visoko značajne rezlike kod svih skorova, odnosno prosečne vrednosti su uvek veće kod studenata ženskog pola u odnosu na muškarce. Istraživanje je pokazalo da je postojao značajan broj studenata sa prisustvom predispitne anksioznosti i koji su zahtevali profesionalnu pomoć i podršku u formiranju, jačanju i proširenju ličnih kompetencija.

Ključne reči: *studenti, anksioznost, stres, ispiti*

ABBREVIATIONS

TAI – Test Anxiety Inventory
TAI-T – Test Anxiety Inventory-Total
TAI-E – Test Anxiety Inventory-Emotionality
TAI-W – Test Anxiety Inventory-Worry
Sd – Standard deviation

UDK: 159.944.4.072-057.875; 616.89-008.441:159.9.072.59(497.11); 613.86-057.875
Ser J Exp Clin Res 2018; 19 (2): 167-174;
DOI: 10.1515/SJECR-2017-0060

Corresponding author: Milica Vasiljevic Blagojevic
High Medical College of professional Studies in Belgrade; sgoras@yahoo.com

INTRODUCTION

Anxiety is a form of pathological fear characterized by a feeling of inner restlessness, timidity and concern. It occurs when there is no real danger or when the emotional response is not commensurate with the experience of risk (1). Fear of exams and tests has the greatest negative effect in the school environment, and usually results in poor success, or achievement of such a level of success below the actual level of ability of an individual (2). Fear of the exam is a result of the need to pass the exam. It can occur as early as in primary or secondary school, at others after a failure at the exam or loss of a year on studies.

Academic success for several decades back has been attracting attention of researchers, resulting in a large number of studies examining various factors contributing to the success and failure of students.

Fear of examination or test anxiety is defined as a complex multidimensional concept that includes cognitive, affective, physiological and behavioral responses to situations that require valuation (3). It is considered a special form of general anxiety that occurs during the exam period. Its signs are stress and anxiety or anguish, which is expressed in the course of the examination, with particular perception of helplessness (4). Since students with test-anxiety in test situations demonstrate greater frequency and intensity of emotional reactions, a test situation can be considered a situation-specific anxiety indication (5). Students with high anxiety during exam experience more frequent and more intense emotional reactions related to anxiety and increased activity of the autonomic nervous system. At the same time, there is a division of attention to the demands posed by the task and cognitive activities such as anxiety and self-criticism, which are irrelevant to the task, but lead to reduced attention and indirectly affect achievement.

During the pre-exam anxiety a set of learning difficulties appears: wandering thoughts, difficulty in memorizing, students just „stare" at the book. Learning becomes difficult and inefficient. Time devoted to learning is used irrationally, as well as student's skills. There is imbalance in the work and rest regime, weariness comes quickly while concentration rapidly declines and attention degrades Although a student continues to learn and tries hardly, the results become weaker, inevitably leading to failure (6).

The consequences of poor coping with the pre-exam anxiety may be the inability to pass an exam, reduced success of studies, leaving the faculty, the decline in self-esteem, unused intellectual potential (7). Self-directed attention, according to Clark and Wells model (8) has a key role in maintaining social anxiety. It is defined as an „awareness of internally created information essential for a person" (9). The content of this awareness may include information about events in the body or awareness of thoughts and emotions including personal beliefs and attitudes. We can say that the focus on oneself is a key factor for test anxiety, too, for assessment of self-efficacy, and an awareness of own automatic thoughts. On the other hand, a small number of students is familiar with the way to deal with pre-exam anxiety (7).

The aim of our study was to investigate the presence of pre-exam anxiety in nursing students and establish the relationship of the pre-exam anxiety and several demographic variables (e.g., gender, age, place of birth, current place of residence, with whom they live, whether they are employed, average family earnings, year of study), objective success (e.g., pass rate at exams, the average score at the end of the year, the possible repeated year of study), and finally subjective perception of academic success (self-satisfaction as a student as well as the importance of grades obtained at the exam). The sample consisted of students of High Medical College of Professional Studies in Belgrade at the study Department for a professional nurse.

MATERIAL AND METHODS

The study was conducted in High Medical College of Professional Studies in Belgrade, during the June exam period in the school year 2014/15. The study included 209 students distributed by years of study, chosen randomly. Of the total number of respondents, the sample consisted of 49.3% (n=103) of the second year students, and 50.7% (n=106) of the third year students. The average age of respondents was 24.11 years (SD=6.26). Among the respondents there were 9.1% (n=19) male students and 90.9% (n=190) female students which is common sex distribution in this kind of a study program. Of the total number surveyed, the 4.3% (n=9) has repeated a year of study. Participants in the research voluntarily and anonymously completed questionnaires right before the test or oral exam lasting 15-20 minutes. All students answered in the same way to the same questions. Though respondents were voluntary, and their participation was anonymous, they were also assured that their responses were confidential. Finally, objective success was evaluated through exam results, grade point average and year of study repeated.

Instruments

Pre-examination anxiety among students was examined using a questionnaire *The Test Anxiety Inventory* – TAI (the questionnaire was used in order to examine the pre-examination anxiety)(10) and it is most often used in surveys on student population. The questionnaire comprises of 20 questions. Respondents answer questions on a four-point Likert scale: 1, never; 2, sometimes; 3, often; 4, always. The minimum score of the questionnaire is 20 and the maximum 80. TAI questionnaire includes subscale scores: overall test anxiety (TAI-T) based on all 20 items, test of anxiety-worry (TAI-W) and the test of emotionality - (TAI-E). Each subscale consists of 8 questions. The other four issues are correlated with the remaining sixteen ones. Questions in the subscale "Worry" refer to the cognitive aspects of test anxiety, that is the experience of anxi-

ety when thinking about the examination outcome in the sense of failure, bad grade and the like. Questions in the subscale "Emotions" refer to the emotional and physiological aspects of anxiety (such as tachycardia, muscle tension, panic) occurring before, during and after the test/exam. Questions in our questionnaires used in this study are very reliable - Cronbach alpha coefficient was 0.94 for the entire questionnaire, and there were no significant changes in any of the individual values of the subscales.

The instrument was applied collectively, during the examination period. First they got the instructions, then filled out indicated questionnaires, and after completion of the test the aim of the study was explained to them.

Statistical data processing

With respect to descriptive and analytical statistical methods were used to analyse the data. The results were shown by arithmetic mean, standard deviation and relative frequencies. For determining the significance of differences, Student's t-tests and ANOVA tests were performed. Statistical processing of demographic data (as categorical variables) was performed using a chi-square test. The statistical significance here was determined at the $p<0.05$ level.

RESULTS

The mean value of the pre-exam anxiety in students of High the Medical College of Professional Studies in Belgrade, measured with the TAI questionnaire was 46.46 (SD=12.62). The mean value of the subscales were, for Emotions = 19.72 (SD=5.73) and Worry = 16.99 (SD=5.03). The results are shown in Table 1.

The results obtained from the TAI questionnaire analysis indicate that there are symptoms of the pre-exam anxiety in relation to sex. Students showed statistically highly significant differences in all scores, and it is due to the fact that the observed scores average values are always higher in female students in relation to men, either by comparing the obtained values of the total questionnaire score t=-3,007; p<0.05 or by comparing the observed Emotionality subscales (t=-2.983; p<0.05) and Worry (t=-2.464; p<0.05). The results are shown in Table 2.

Using a t-test to compare questionnaire scores, we showed that pre-exam anxiety symptoms were significantly more pronounced among second-year students than among third-year students for each of the se subscales. This was true in relation to questionnaire total scores (t= 2.72; p<0.01) and in relation to the Emotionality subscale (t=4.15; p<0.01) and the Worry subscale (t=3.82; p<0.01). When comparing the scores of second- and thirdyear students' scores, statistically significant differences between them were observed with respect to the questionnaire's score overall (mean=49.73, SD=11.49; p<0.01) the Worry subscale (mean=17.94, SD=4.64; p<0.01) and the Emotionality subscale (mean=21.33; SD=5.35; p<0.01) among the second- and third- year students. The results are shown in Table 3.

Table 1. Pre-examination anxiety measured by the TAI questionnaire

Subscale	Mean	SD
TAI-T/ Total score	46.46	12.62
TAI-W / Worry	16.99	5.03
TAI-E / Emotionality	19.72	5.73

Table 2. Pre-examination anxiety obtained by comparing students by sex

Subscale	Gender	Mean	SD	P
TAI-T/ Total score	Male	38.32	11.21	.003
	Female	47.28	12.49	
TAI-W / Worry	Male	14.32	4.45	.015
	Female	17.26	5.02	
TAI-E / Emotionality	Male	16.05	4.68	.003
	Female	20.09	5.71	

Using t-tests, we found that there were no statistically significant differences in exam anxiety intensity with respect to students' place of birth. Among average values for each of the three scores we see that the averages for all scores were higher for students born in Belgrade than for those born in the interior of Serbia; however, therese were not statistically significant differences, whether at the level of the entire questionnaire ($t = 1.901$; $p> 0.05$), nor for the Emotionality ($t = 1.561$; $p <0.05$) and Worry subscales ($t =1.800$; $p> 0.05$). The results are shown in Table 4.

A comparison of score averages and their relation to students' current residence showed that average values for students who lived in suburbs were always higher; students who lived in the countryside were always the lowest; while, while students living in the city had scores in the middle across all of the subscales.

The results of the ANOVA test using Tukey comparisons indicate that statistically significant differences were not determined when comparing the results from the entire questionnaire (F=.131; p>0.05), or from the Emotionality subscales y(F=.037; p>0.05) or the Worry subscales

Tablea3. The intensity of the pre-exam anxiety in relation to the year of study

Subscale	*Year of study*	Mean	SD	P
TAI-T/ Total score	II	49.74	11.49	.000
	III	43.25	12.90	
TAI-W / Worry	II	17.94	4.63	.007
	III	16.07	5.26	
TAI-E / Emotionality	II	21.34	5.36	.000
	III	18.16	5.68	

Table 4. Average values for the scores in relation to the student's place of birth

Subscale	place of birth	Mean	SD	P
TAI-T/ Total score	Belgrade	49.12	12.52	.05
	Inland	45.43	12.56	
TAI-W / Worry	Belgrade	18.00	4.95	.07
	Inland	16.62	5.03	
TAI-E / Emotionality	Belgrade	20.71	5.73	.12
	Inland	19.34	5.71	

Table 5. Mean values of observed scores in relation to a student's current residence

Subscale	Residential status	Mean	SD	P
TAI-W / Worry	city	16.91	5.10	
	suburb	17.56	4.85	.759
	village	16.62	4.94	
TAI-E / Emotionality	city	19.69	5.72	
	suburb	19.97	5.90	.964
	village	19.62	5.97	
TAI-T / Total score	city	46.28	12.64	
	suburb	47.47	12.84	.877
	village	46.06	12.77	

(F=.276; p>0.05). Students with the most prominent symptoms of pre-exam anxiety lived in suburbs, while those with the least lived in the countryside. This is true even though the score averages were about the same for all three types of settlements where the students lived. The results are shown in Table 5.

A comparison of score averages and their relation to students' current residence showed that average values for students who lived in suburbs were always higher; students who lived in the countryside were always the lowest; while students living in the city had scores in the middle across all of the subscales.

The results of the ANOVA test using Tukey comparisons indicate that no statistically significant differences existed in results for the entire survey (F = .947; p> 0.05) or for those associated with the Emotionality (F = 1.089; p <0.05) and Worry subscales (F = 1.037; p <0.05). Students with the most prominent pre-exam anxiety symptoms lived with a boyfriend/girlfriend, and those with the least anxiety with a roommate in a rented apartment. The results are shown in Figure 1.

Table 6. Average values for the scores with respect to employment

Subscale	Are you employed	Mean	SD	P
TAI-T/ Total score	Yes	46.04	12.26	.728
	No	46.68	12.85	
TAI-W / Worry	Yes	16.75	4.93	.610
	No	17.12	5.10	
TAI-E / Emotionality	Yes	19.50	5.58	.679
	No	19.85	5.83	

A comparison of averages for the three observed scores in relation to students' employment status (i.e., whether employed or not) showed no statistically significant differences in any questionnaire scores, that is average values were not significantly different with respect to candidate's employment status (i.e., whether he/she was employed or not).

Comparing the scores from the TAI questionnaire with the help of t-tests, we see no significant differences in the intensity of the pre-exam anxiety between students who were employed and those who were not. However, students who were not employed showed pronounced symptoms of anxiety compared to those who worked, yet statistically significant differences were not found when comparing responses drawn from the entire questionnaire (t = -0.348, p> 0.05), nor when comparing scores on either the Emotionality (t = -0.414, p> 0.05) or Worry subscales (t = -0.510, p> 0.05). The results are shown in Table 6.

Next, a comparison of the three observed averages with respect to whether a candidate usually passed their exams the first time they are administered showed a statistically highly significant relationship with academic achievement. This relationship is a consequence of the fact that all observed mean scores were always higher among students who tended to fail the same exam and whose who tended to pass their exams the first time they take it.

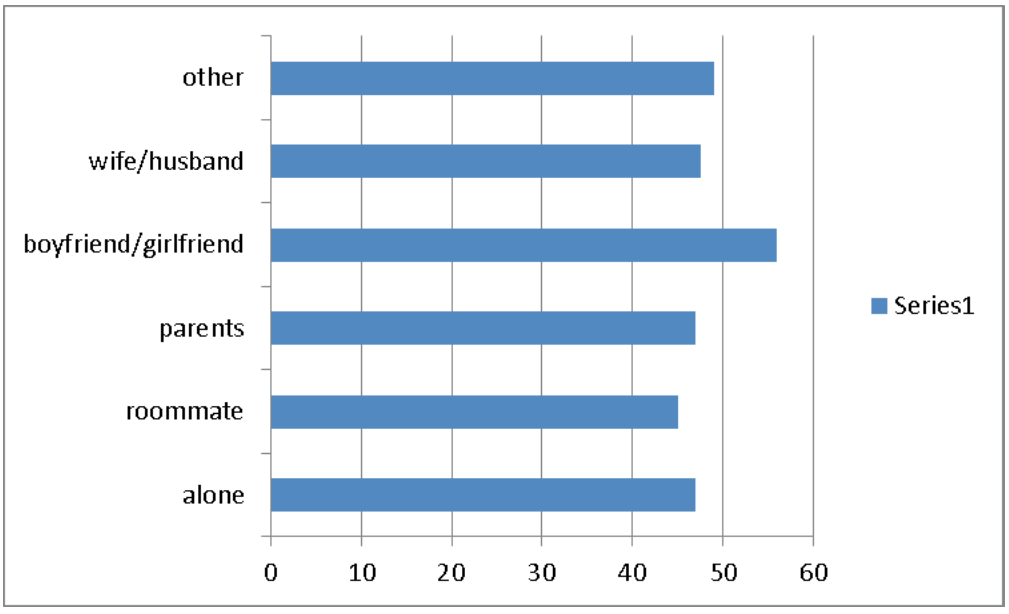


Figure 1. The values for the scores in relation to with whom the student is currently living

Table 7. Average values of the scores compared to the pass rate at exams

Subscale	Pass rate at exams	Mean	SD	P
TAI-W / Worry	I mainly pass the exam the first time I take it	15.72	4.74	
	Failure happens to me, but not the same exam several times	18.61	4.89	
	I repeatedly fail the same exams	20.75	4.34	.000
	Total	17.00	5.03	
TAI-E / Emotionality	I mainly pass the exam the first time I take it	18.92	5.70	
	Failure happens to me, but not the same exam several times	20.77	5.63	
	I repeatedly fail the same exams	21.94	5.53	.029
	Total	19.73	5.74	
TAI-T / Total score	I mainly pass the exam the first time I take it	43.77	12.22	
	Failure happens to me, but not the same exam several times	49.92	12.31	
	I repeatedly fail the same exams	54.19	11.09	.000
	Total	46.46	12.62	

With respect to the students' exam pass rate, results of the ANOVA test using Tukey comparisons indicate statistically significant differences between the results of the entire survey ($F = 8.958$; $p < 0.001$) as well as and between the scores on the Emotionality ($F = 3.607$; $p < 0.05$) and Worry subscales ($F = 13.268$; $p < 0.001$). Students with the most prominent symptoms of the pre-exam anxiety repeatedly failed the same exam, while the students with the lowest anxiety passed the exams on their first efforts. The results are shown in Table 7.

There was no statistically significant difference in the intensity of symptoms among pre-exam anxiety students who have repeated a year of study.

Table 8. The intensity of the pre-exam anxiety in relation to the repeated year of study

Subscale	Have you ever repeated a year of study	Mean	SD	P
TAI-T/ Total score	Yes	49.78	12.78	.422
	No	46.31	12.63	
TAI-W / Worry	Yes	19.33	5.39	.155
	No	16.89	5.00	
TAI-E / Emotionality	Yes	19.78	5.04	.979
	No	19.72	5.78	

Using the t-test to compare TAI questionnaire parameters of students who had andwith students who had not, repeated a year of study, showed no significantly different intensity of pre-exam anxiety. Students who had repeated years showed pronounced symptoms of anxiety compared to those who studied regularly, but a statistically significant difference was not observed when comparing their results on the entire questionnaire ($t = 0.805$; $p > 0.05$), or on their scores from the Emotionality ($t = 0.027$; $p > 0.05$) or Worry subscales ($t = 1.429$; $p > 0.05$). The results are shown in Table 8.

Subjective perception was measured through the self-satisfaction of oneself as a student and the importance of the obtained evaluation.

Comparing averages for the three aspects in relation to how much the candidate is pleased with him/herself as a student confirmed the existence of statistically significant differences in the total scores and in the worry scores, while emotionality scores did not show statistical significance.

The results of ANOVA test with Tukey comparison indicate statistically significant differences when comparing the obtained results of the entire survey ($F = 2.750$; $p < 0.05$) as well as at the Worry subscale ($F = 4.180$; $p < 0.05$). A statistically significant difference was not found only on the Emotions subscale ($F = 1.849$; $p > 0.05$). Students with the most prominent symptoms of pre-exam anxiety are also the least satisfied with themselves as students. The results are shown in Table 9.

Table 9. Pre-examination anxiety was measured through the self-satisfaction as a student

Subscale	Pass rate at exams	Mean	SD	F	P
TAI-W / Worry	Satisfied, I learn the best I can	15.83	5.10		
	Partially satisfied, I could study better	17.31	4.76		
	Dissatisfied, but I do not have the will to do better than this	19.40	5.76	4.18	.007
	Dissatisfied, I try much, but to no success	29.00	.		
	Total	17.00	5.03		
TAI-E / Emotionality	Satisfied, I learn the best I can	19.12	6.05		
	Partially satisfied, I could study better	19.93	5.48		
	Dissatisfied, but I do not have the will to do better than this	19.80	6.25	1.85	.139
	Dissatisfied, I try much, but to no success	32.00	.		
	Total	19.73	5.74		
TAI-T / Total score	Satisfied, I learn the best I can	44.35	13.12		
	Partially satisfied, I could study better	47.11	12.08		
	Dissatisfied, but I do not have the will to do better than this	48.90	13.16	2.75	.044
	Dissatisfied, I try much, but to no success	76.00	.		
	Total	46.46	12.62		

DISCUSSION

The High Medical School of Professional Studies in Belgrade is the only school in Serbia with several decades of tradition where, in addition to nurses, eight other professions are educated. This school is one of the most prestigious institutions of higher education, as evidenced not only by the school's admission selectivity but also by the high percentage of graduates employed. The challenges of modern medical education, along with the plans and programmes of the medical school itself, entail numerous obligations and demanding examinations that affect students' mental and emotional status. Intensive multi-year education and the high standards of medical sciences in general suggest that medical sciences students are of a type, one with a high degree of pre-exam anxiety (10).

As a result, there is a need for the continual evaluation of pre-exam anxiety and its effects on the student population. There are numerous global studies on the subject of pre-exam anxiety in nursing students, and most of them confirm the existence of this phenomenon, only to greater or lesser degrees (11, 12). However, these studies used different research methodologies, which complicates the comparison of the results of different studies.

With the objective of determining the presence of the pre-exam anxiety, our analysis of the TAI questionnaire results showed that nursing students were statistically significant for pre-exam anxiety across all subscales. Preexam anxiety symptoms related to the sex of the candidates showed highly significant statistical differences in all scores because the observed average scores were always higher in female students relative to men. This was true when comparing questionnaire's total score or when comparing the observed Emotions and Worry subscales. The mean values obtained by the TAI questionnaire for male and female students and for the Emotionality and Worry subscales appeared similar to student population normative data obtained by the questionnaire's author (13).

The results of pre-exam anxiety showed that female students felt greater discomfort and worried more than male ones. They also confessed to having poorer concentration on tests, were and were more likely than men to suggest that thoughts about the exam itself could paralyze them. Female students were also likely to claim that they had more prominent physical symptoms prior to examinations (i.e., rapid heartbeat, stomach discomfort, sweating and flushing). Similar results were obtained from other

studies using the same methodology. Reteguiz found that female medical students had statistically significant higher scores on the TAI questionnaire and on both subscales (14). Some authors explain this difference as reflecting the fact that females have a greater willingness to admit their anxiety than males (15). However, it is possible that women students have higher levels of anxiety than their male counterparts.

In our research, pre-exam anxiety symptoms are significantly more pronounced across all subscales for second-year students compared to third-year students.

Students with the most prominent pre-exam anxiety symptoms live in suburbs, while those with the least prominent ones live in the countryside. However, the average scores across all three development types are about the same. Based on these results, we can conclude that respondents' place of residence has no particular impact on pre-exam anxiety. Nevertheless, the actual student experiences suggest that those who study outside of their place of residence are under greater pressures in light of the higher total living costs during their studies.

Students with the most prominent symptoms of pre-exam anxiety lived in partnerships with a boyfriend/girlfriend, while those living with a roommate in a rented apartment had the least anxiety.

This result can be interpreted only partially because there are no data on whether those living with roommates also study with them. We can only assume that these roommates are also students, that they are learning together and sharing their experiences and that they have similar ways of studying, ultimately "reducing" pre-exam anxiety. It would be extremely important to include this parameter in a future studies.

Comparing TAI questionnaire parameters using a t-test showed that employed students had no significant differences pre-exam anxiety intensity than unemployed students. Unemployed students showed pronounced symptoms of anxiety compared to those who worked, and a statistically significant difference was determined based on results from the entire questionnaire. We can assume that employed students are more burdened by everyday business and professional activities, yet they are also more experienced with techniques related to overcoming anxiety, and thus show reduced levels of pre-exam anxiety.

Regarding student performance on examinations, results indicate highly significant statistical differences when comparing the results from the entire survey and when comparing scores from the Emotionality and Worry subscales.

Students with the most prominent pre-exam anxiety symptoms happened to repeatedly fail the same exam, while students the lowest anxiety levels were those who had passed the exams the first time taking them.

This result is expected. Given the high levels of anxiety associated with the exam experience, the more frequent and more intense emotional reactions related to anxiety and the increased activity of the autonomic nervous system, it is not surprising that students are being trippedup during specific exam activities. As mentioned above, Spielberger and Vagg stipulated that multiple concerns and thoughts directed at oneself can emerge simultaneously, and though irrelevant to the task, they can influence the operation of one's attention and performance on exams, indirectly affecting achievement (5). The link between anxiety and test performance was also recognized in Def-

fenbacher's research, distinguishing the different connections between the components of care and academic success, in both high school pupils and students (16).

On the other hand, there was no statistically significant difference in intensity of pre-exam anxiety symptoms between students who had repeated a year of study and those who entered it for the first time. Students with the most prominent pre-exam anxiety symptoms were those least satisfied with themselves as students. This is a consequence of the fact that observed mean scores were always higher in students who claimed to be neither happy with themselves, nor with the academic success they had achieved. Indeed, scores were always lowest among students who claimed to be happy with themselves and who had done their best. This finding is also not surprising. According to several authors, the consequences of overcoming pre-exam anxiety poorly may be an inability to study, reduced academic performance, reduced self-esteem and unused intellectual potential (7). Our results are consistent with these earlier findings.

CONCLUSION

The high level of pre-exam anxiety among medical students stems from several years of intensive training in the medical sciences. More than in other fields, these students deal with quick and intensive changes daily and with the constant introduction of new and higher standards. Our motive to analyze is pre-exam anxiety among the High Medical School students was rooted in our awareness of the high degree of pre-exam anxiety in nursing students around the world. Previous researches results, regardless of methodologies used, confirm our own findings: there is a high degree of pre-exam anxiety in nursing students.

The higher level of pre-exam anxiety described in our study was evident in the female students. They

stated (more frequently than their male counterparts) that they had low concentration for the exams and that the very thought of the exam could paralyze them. Additionally, female students had more pronounced physical symptoms prior to an examination with respect to a more rapid heartbeat, stomach discomfort, sweating and flushing. Our research has also shown that a students' place of residence has no particular impact on pre-exam anxiety; however, actual student experiences suggest that students who study outside of their place of residence are under greater pressures as their total living costs during their studies is correspondingly higher.

A very interesting and important finding in this research was that students with the most prominent symptoms of pre-exam anxiety tend to live with a boyfriend/girlfriend, while those with the least anxiety tended to live with a roommate in a rented apartment. We could only partially interpret this finding asbecause we had no data on whether those living with a roommate were also studying with them. We can only assume that roommates are also learning together, sharing experiences and studying in similar ways, ultimately reducing any pre-exam anxiety. It would be extremely important to include this parameter in subsequent research.

In addition, unemployed students have demonstrated more pronounced anxiety symptoms compared to the employed ones. However, a statistically significant difference was not found when the results from the entire questionnaire were compared. Employed students are probably more burdened by everyday business and professional activities, yet also more experienced in techniques for overcoming anxiety and hence have reduced levels preexam anxiety.

Students at the High Medical College of Professional Studies in Belgrade examined here had very little theoretical knowledge of methods and techniques for overcoming pre-exam anxiety. This work's contribution lies in its focus on the "initial state", identifying the high degree of pre-exam anxiety in nursing students and its proposal for the improvement of this aspect of their education. Their education should entail the expansion individual competences, a capacity to accept responsibility for their own lives, and for enriching the repertoire of adaptive and developmentally useful responses to serious and stressful situations.

REFERENCES

1. Latas M., Lečić Toševski D. Anxiety disorders. In: Jašović Gašić M, Lečić Toševski D. (2007). A textbook for psychiatry for medical students. Belgrade: Medical faculty. 156-169.
2. Birenbaum, M., Nasser, F. (1994). On the relationship between test anxiety and test performance. Measurement and Evaluation in Counseling and Development, 21, 293-302.
3. Hong, E., Karstensson, L. (2002). Antecedents of state test anxiety. Contemporary Educational Psychology. 27. 348-367.
4. Dykeman, B.F. (1994). The effects of motivational orientation, self-efficacy and feedback condition on test anxiety. Journal of instructional Psychology. 21. 114-120.
5. Spielberger, C.D. , Vagg, P.R. (1995). Text anxiety: transactional process model. U C.D. Spielberg i P.R. Vagg (Ur.), Test anxiety: theory, assessment and treatment (3-15). Washington: Taylor & Francis.
6. Erić, Lj. (1977) Fear of exam: his nature and healing. Medical book. Belgrade. 55.
7. Neuderth S., Jabs B., Schimdtke A. (2009). Strategies for reducing test anxiety and optimizing exam preparation in German university students: a prevention-oriented pilot project of the University of Würzburg. J Neural Transm. 116(6). 785-790.
8. R.G. Heimberg, M.R. Liebowitz, D.A. Hope, F.R. Schneier (1995). Social Phobia: Diagnosis, Assessment, and Tretment. (p.p. 69–93). New York: The Guilford Press.

9. Ingram, R.E. (1990). Self-focused attention in clinical disorders: Review and conceptual model. Psychological Bulletin. 107. 156-176.
10. Rosario P., Nunez JC, Salgado A., Gonzalez-Pienda JA, Valle A., Joly C. (2008). Test anxiety: associations with personal and family variables. Psicothema. 20/4. 563-570.
11. Nitasha Sharma, Amandeep Kaur. (2011) Factors associated with stress among nursing students. Nursing and Midwifery Research Journal. 7/1.
12. April L. Shapiro. (2014). Test anxiety among nursing students: A systematic review. Teaching and Learning in Nursing. 9/4. 193–202.
13. Spielberger, C.D. Gonzales, H.P. Taylor, C.J. Anton, E.D. Algaze, B. Ross,G.R. et al. (1980) Manual for the test Anxiety Inventory "Test Attitude Inventory". Redwood City, CA: Consulting Psychologists Press.
14. Chapell MS, Blanding B, Silverstein ME, et al. (2005). Test anxiety and academic performance in undergraduate and graduate students. Journal of Educational Psychology. 97/2. 268-274.
15. Rohe DE, Barrier PA, Clark MM, Cook DA, Vickers KS, Decker PA. (2006). the benefits of pass-fail grading on stress, mood, and group cohesion in medical students. Mayo Clin. 81/11. 1443-1448.
16. Deffenbacher, J.L. (1980). Worry and emotionality in test anxiety. U I.G. Sarason (Ur.), Test anxiety: theory, research and applications. (p.p.111-128). New Jersey: Lawrence Earlbaum Associates, Inc.

GENETIC METHODS FOR DETECTING ASTROCYTES, NEURONS AND NEUROGENESIS

Natalia Nikolaevna Shusharina[1], Ekaterina Vladimirovna Silina[2], Aleksandr Alexandrovich Vasilyev[1], Irina Nikolaevna Dominova[1], Victor Aleksandrovich Stupin[3], Tatiana Georgievna Sinelnicova[2], Egor Borisovich Sotnikov[1], Andrey Victorivich Turkin[1], Maksim Vladimirovich Patrushev[1]
[1]Institute of Life Sciences; Immanuel Kant Baltic Federal University (IKBFU), Kaliningrad, Moscow, Russia
[2]Department of Human Pathology, I.M. Sechenov First Moscow State Medical University (Sechenov University), Moscow, Russia
[3]Department of hospital surgery №1; N.I. Pirogov Russian National Research Medical University (RNRMU), Moscow, Russia

GENETIČKE METODE DETEKCIJE ASTROCITA, NEURONA I NEUROGENEZE

Natalia Nikolaevna Shusharina[1], Ekaterina Vladimirovna Silina[2], Aleksandr Alexandrovich Vasilyev[1], Irina Nikolaevna Dominova[1], Victor Aleksandrovich Stupin[3], Tatiana Georgievna Sinelnicova[2], Egor Borisovich Sotnikov[1], Andrey Victorivich Turkin[1], Maksim Vladimirovich Patrushev[1]
[1]Institut za nauku Immanuel Kant, Balticki drzavni univerzitet (IKBFU), Kaliningrad, Moskva, Rusija
[2]Odsek za humanu patologiju, I.M. Sechenov Prvi Moskovski državni medicinski univerzitet (Univerzitet Sechenov), Moskva, Rusija
[3]Odsek za hirurgiju №1; N.I. Pirogov Ruski nacionalni istraživački medicinski univerzitet (RNRMU), Moskva, Rusija

Received / Primljen: 23.04.2018. Accepted / Prihvaćen: 30.04.2018.

ABSTRACT

Two sets of reactants for modelling neurogenesis (SRMN) were developed based on the designed and tested genetic structures of lentiviral vectors. SRMN-1 contains the genetic construct LVV-GFAP-GCaMP3 and is intended for cell-specific transduction in astroglia cells. SRMN-2 contains the genetic construct LVV-PRSx8-TN-XXL and is intended for the phenotype-specific transduction in neurons. The present study examined SRMN-1 and SRMN-2 samples and assessed their efficiency in vitro and in vivo in Norvegicus rats. Specificity to particular cell types for all SRMN samples exceeded 97%. The number of induced signalling cascades was determined via activation of intracellular ingsignalling cascades in neurons and astrocytes (purinergic receptors and β-adrenoceptors). The results demonstrated dynamic recording of fluorescent signals and a two-fold increase in intensity after addition of the activator in all samples. The experimental SRMN samples revealed successful and stable transfection of catecholaminergic neurons and astrocytes, data on transfection efficiency, specificity of the developed genetic structures of SRMN, and calcium dynamics in transfected neurons and astrocytes.

These results confirm the crucial role of astrocytes in ensuring neurogenesis. The results in pure cell culture (in vitro) were identical to the in vivo results in animals.

Keywords: *neurogenesis astrocytes, neurons, signalling cascades, genetic structure, viral vector.*

SAŽETAK

Dva seta reaktanata za modeliranje neurogeneze (SRMN) su razvijena i bazirana na projektovanim i testiranim genetskim strukturama zasnovanim na lentiviralnom vektoru. SRMN-1 sadrži genetski konstrukt LVV-GFAP-GCaMP3, namenjen je za ćelijsko specifičnu transdukciju ćelija astroglije. SRMN-2 sadrži genetski konstrukt LVV-PRSk8-TN-KSKSL, namenjen je za fenotipski specifičnu transdukciju neurona. Ova studija je imala za cilj da prouči efekte i efikasnost SRMN-1 i SRMN-2 uzoraka u in vitro i in vivo uslovima na pacovima Norvegicus soja. Specifičnost određene vrste ćelija za sve uzorke SRMN-a prevazišla je 97%. Broj indukovanih signalnih kaskada određen je aktiviranjem intracelularnih signalnih kaskada neurona i astrocita (purinergičkih receptora i B-adrenoceptora). U studiji su prikazani rezultati dinamičkog snimanja fluorescentnog signala, čiji je intenzitet nakon dodavanja aktivatora povećan više od dva puta u svim uzorcima. Rezultati korišćenja metode procene efikasnosti eksperimentalnih SRMN uzoraka su sledeći: uspešna i stabilna transfekcija kateholaminergickih neurona i astrocita; dobijanje eksperimentalnih podataka o efikasnosti transfekcije, specifičnosti razvijenih genetičkih struktura koje su deo SRMN; i dobijanje eksperimentalnih podataka o dinamici kalcijuma kod transfektovanih neurona i astrocita. Rezultati studije potvrđuju ključnu ulogu astrocita u osiguranju neurogeneze. Dobijeni rezultati na čistoj kulturi ćelija (in vitro) su identični onima dobijenim in vivo na životinjama.

Ključne reči: *neurogeneza astrocita, neuroni, signalne kaskade, genetička struktura, virusni vektor.*

sciendo

UDK: 612.822.08 / Ser J Exp Clin Res 2018; 19 (2): 175-184
DOI: 10.2478/SJECR-2018-0013

Corresponding author: Ekaterina Vladimirovna Silina, MD, PhD,
Department of Human Pathology, I.M. Sechenov First Moscow State Medical University (Sechenov University),
8 Trubetskaya Str, Moscow, 119991, Russia; mail: silinaekaterina@mail.ru

INTRODUCTION

The incidence of cerebral pathology has progressively increased in recent decades because of the increasing life expectancy of the human population. This increase especially applies to disabling pathologies, such as apoplexy and neurodegenerative diseases (1, 2). One possible reason for the lack of significant progress in treatment may be that the primary vector of investigations of the pathogenesis of neurological diseases is neurons. This focus may be due to the ambiguous effects of neuroprotective therapy (3).

Glia represent greater than 50% of cells in the brain, and these cells outnumber neurons (4). Astrocytes are the most common and important glial cells, account for 30-40% of the total number of brain cells (5). Astrocytes primarily form the haematoencephalic barrier and provide an adequate supply of nutrition to neurons. These functions are disrupted in Parkinson's disease (6). Astrocytes provide structural and metabolic support, regulate synaptic transmission and water transport, and play an active role neuronal plasticity (7, 8). Therefore, astrocytes may underlie diseases of the central nervous system.

The development of genetic engineering and genetics in neurosciences yielded data that confirmed this assumption. For example, scientists examined the expression levels of known key genes of Parkinson's disease in human and mouse astrocytes and neurons and found that monogenic mutations were expressed in astrocytes at levels comparable to or, in some cases, higher than neurons (9, 10). New scientific evidence confirmed the important roles of astrocytes in the development of various diseases, including neurodegeneration (11, 12). However, this role of astrocytes remains controversial in scientific circles.

Fundamental research in this area is required to resolve this controversy, and genetic methods of research appear promising.

The present study developed genetic methods of registering and correcting neurogenesis disruptions in the brain using viral vectors and assessed the efficiency of the developed methods *in vitro* and *in vivo.*

MATERIALS AND METHODS

The first experiments used two sets of reactants for modelling neurogenesis (SRMN) based on the designed and tested genetic structures.

The set of reactants for modelling neurogenesis No. 1 (SRMN-1) contained the genetic construct LVV-GFAP-GCaMP3 based on the lentiviral vector, which is specific for astroglia. These constructs were maintained in DMEM cultural medium with 10% foetal bovine serum. SRMN-1 carries a genetically encoded calcium indicator (GCaMP3) under the control of cell-specific promoter of glial fibrillar acid protein (GFAP), which is intended for cell-specific transduction in astroglia cells (Fig. 1).

A set of reactants for modelling neurogenesis No. 2 (SRMN-2) contained the genetic construct LVV-PRSx8-TN-XXL based on the lentiviral vector, which is specific for catecholaminergic neurons. These cells were maintained in DMEM culture medium with 10% foetal bovine serum. SRMN-2 is intended for phenotype-specific transduction in neurons (Fig. 2).

These genetic structures exhibit the following characteristics: lentiviral vector; visualized chemically; size of the experimental sample not greater than 25,000 nucleotides/pairs of nucleotides; auxiliary components of genetic constructs not greater than 370 nucleotides/pairs of nucleotides; specificity to certain cell type not less than 97%; only transferred cells exhibited differentiation (SRMN No. 1 for astrocytes, SRMN No. 2 for catecholaminergic neurons); and the titer of the genetic constructs not less than 0.5×10^6 T.U./ml (T.U. – transducing unit).

The SRMN exhibited the following primary properties: (1) ability to integrate into the genome of the target cell to ensure permanent expression of the transgene; (2) ability to transfect differentiated cells; (3) wide tropism due to packing of vesicular stomatitis into the shell of the virus; and (4) low immunogenicity when used in animals.

The developed methods of registration and correction of neurogenesis abnormalities in the brain based on functional and/or genetically coded sensors included the following technological procedures:

1. Preparation of fragments for cloning (amplification of fragment of post-transcriptional regulatory element, amplification of fragment of marker gene, amplification of cell-specific promoter);
2. Cloning fragments into a vector (selecting target fragments in the DNA sequence, fractionation of fragments by the method of gel electrophoresis and purification, cloning of purified fragments into a vector);
3. Producing vectors (cultivation of HEK293T cell culture, preparation of the vector for transfection, seeding cells into Petri dishes, transfection, vector production, isolation and purification of the vector); and
4. Concentrating the vector (concentrating vectors via centrifugation, storage), filling, labelling and packaging.

The second stage of work performed experimental studies using the developed SRMN samples, followed by assessment of their efficiency, in vitro and in vivo in 24 rats Rattus Norvegicus.

Each experiment examined 3 samples of SRMN-1 and 3 samples of SRMN-2.

The experimental design for samples of SRMN to assess efficiency in the *in vitro* experiments was based on a combination of methods for isolating and cultivating astrocytes and neurons in the brain, their transfection with viral constructs, and recording the fluorescent images for subsequent assessments of performance.

The model was pure cultures of astrocytes and neurons to assess the efficiency of genetic structures because fluorescent protein expression (GCaMP3 and TN-XXL)

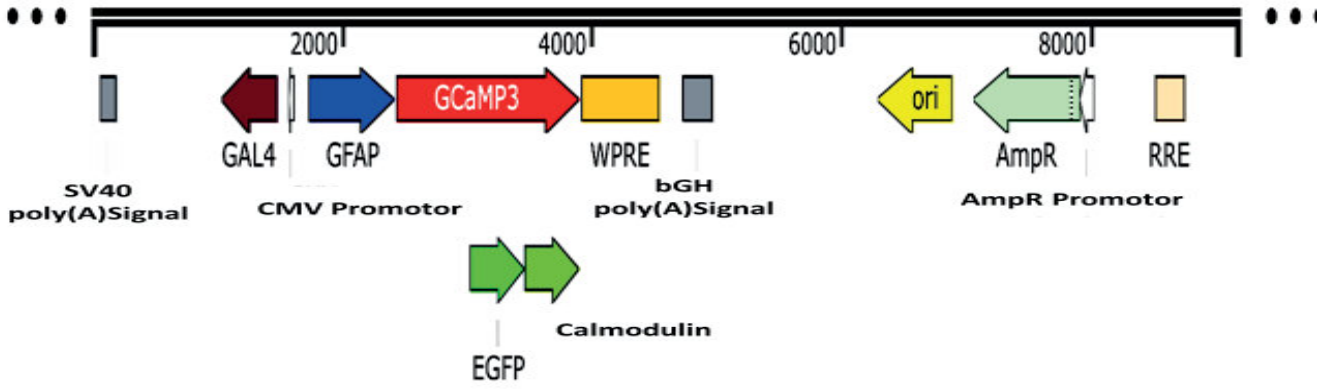


Figure 1. The diagram of the genetic construct LVV-GFAP-GCaMP3 carrying genetically encoded calcium indicator GCaMP3 under the control of cell-specific promoter glial fibrillose acidic protein (GFAP): SV40 poly(A) signal is a signal of SV40 polyadenitis formation; GAL4 is a chimeric transactivator that includes parts of transactivatorg domain of mice protein NF-κBp65 fused with a DNA-binding domain of yeast protein GAL4; CMV is the minimal promoter of human cytomegalovirus; GFAP is the compact promoter of glial fibrillary acidic protein; GCaMP3 is a sequence that encodes genetically encoded calcium indicator GCaMP3; EGFP is reinforced green fluorescent protein; Calmodulin is a calcium binding protein; WPRE is a post-transcription regulatory element of Groundhog hepatitis B virus; bGHpoly(A) signal is a signal of polyadenylation; ori is the high-scale start point of ColE1/pMB1/pBR322/pUC plasmids replication; AmpR is a sequence that provides resistance to ampiciline, carbencillin, and other bonded antibiotics; AmpR promoter is a promoter that ensures resistance to ampicillin; bom is the basic cell region of the plasmid pBR322; RRE is the rev-responsive element of HIV-1, which provides a Rev-responsible export of mRNA from the nucleus into the cytoplasm

could only be analysed qualitatively and quantitatively in the monolayer cell cultures. The detailed protocols for all stages of this work were developed.

The following protocol was used to obtain pure cultures of neurons and astrocytes: obtaining a mixed suspension and mixed culture of brain cells from rats; and purification to obtain pure cultures of neurons and astrocytes. Cell cultures were further investigated using SRMN-1 and SRMN-2 to assess specificity and identify induced cascades (Figure 3).

Cell cultures were transfected in standard growth medium with the addition of an aliquot of sample SRMN to neurons or astrocytes. Laser scanning confocal microscopy was used to assess the efficiency of SRMN samples. This method allowed the recording of fluorescent protein sensor expression (GCaMP3 and TN-XXL) with high precision.

Specificity of genetic constructs to certain cell types was determined via in vitro transfection of pure rat brain cell cultures, staining for cell-specific markers, and recording of the fluorescent signal using an LSM 780 confocal microscope (Carl Zeiss).

Astrocyte primary culture was obtained from 3-day-old rats. The cells were grown in the Dulbecco modified Eagle's medium (DMEM) (with 4.5 g/l glucose, glutamine, and pyruvate) ("GIBCO", UK) with 10% foetal bovine serum ("GIBCO", UK).

Neuronal primary cultures were obtained from 1-day-old rats. The cerebral cortex was extracted on ice in Krebs-Ringer solution (135 mM of NaCl, 5 mM of KCl, 1 mM of $MgSO_4$, 0.4 mM of KH_2PO4, 15 mM of glucose, and 20 mM of HEPES, pH 7.4), trypsinized in a 0.08% solution of trypsin-EDTA for 10 min at 37°C, and ground in a 0.008% solution DNAaza-I containing 0.05% of a trypsin inhibitor. Cells were resuspended in minimal Eagle's medium with Earl's salts (BME) containing 10% of heat-inactivated foetal bovine serum ("GIBCO", Germany), 2 mM of GlutaMAX-I, and gentamicin (100 μg/ml). Cells were seeded on 24-hole tablets covered with poly-L-lysine at a density

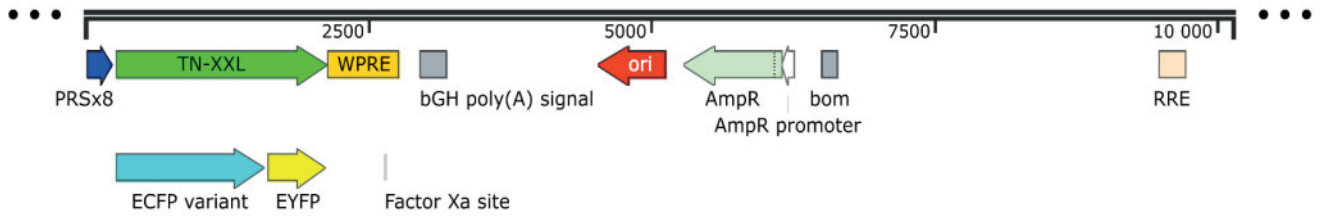


Figure 2. The diagram of genetic construct LVV-PRSx8-TN-XL, TN-XXL under the control of artificial specific neuronal promoter (PRSx8): PRSx8 is an artificial phenotype-specific neuronal promoter; TN-XXL is a sequence that encodes the genetically coded calcium indicator TN-XXL; ECFP variant is enhanced blue fluorescent protein; EYFP is enhanced yellow fluorescent protein; WPRE is post-regulatory element of the Groundhog hepatitis B virus; bGH poly(A) signal is a polyadenylation signal; ori is the high-scale point of plasmids ColE1/pMB1/pBR322/pUC replication start; AmpR is the sequence that ensures resistance to ampicillin, carbenicillin, and other bonded antibiotics; AmpR promoter is a promoter that ensures resistance to ampicillin; bom is the basic cell region of the plasmid pBR322; RRE is the rev-responsive element of HIV-1, which provides a Rev-responsible export of mRNA from the nucleus into the cytoplasm

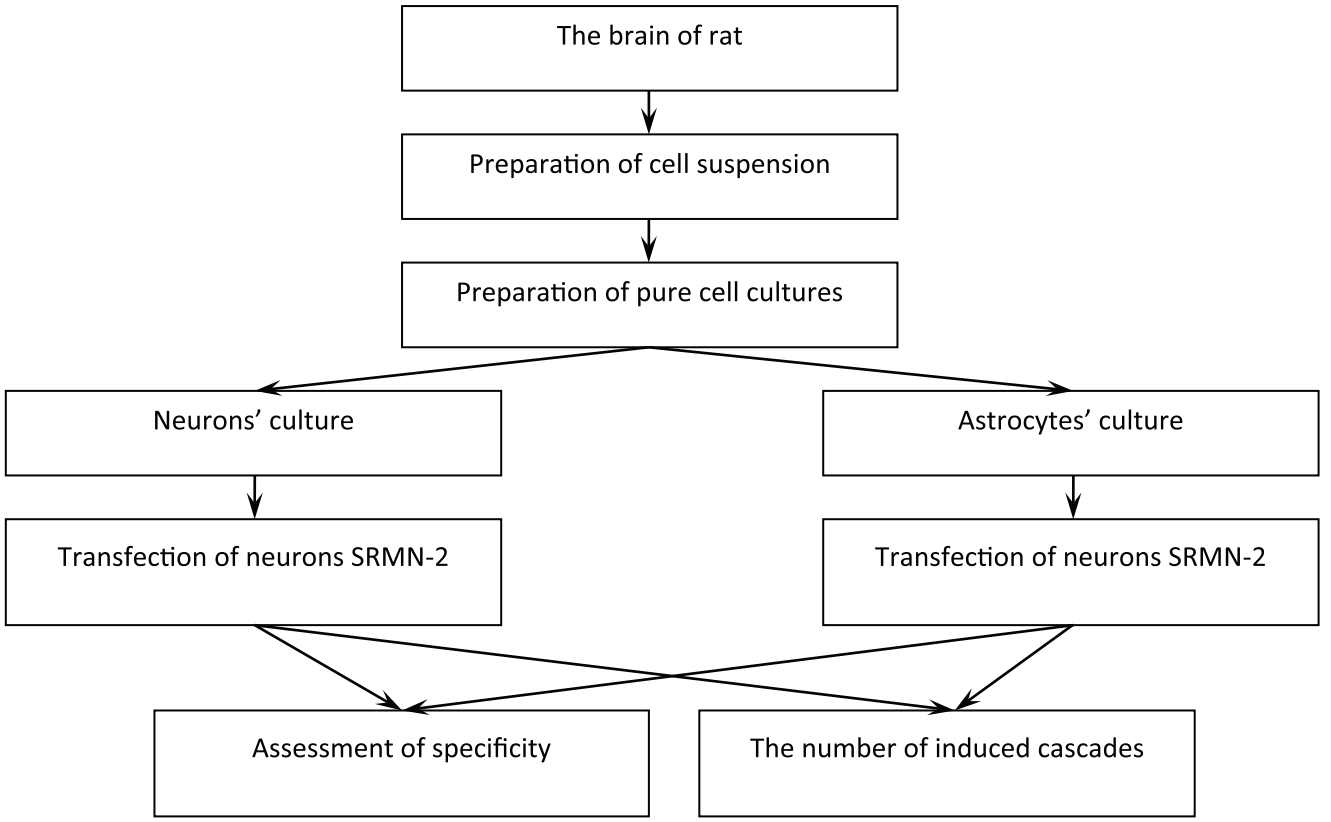


Figure 3. The plan of studying experimental SRMN samples for assessing their efficiency in the in vitro experiments

of 2.5×10^5 cells/cm^2. BME medium was replaced after 2.5 hours with the Neurobasal-A medium, containing 2 mM of GlutaMAX-I, component B-27 and gentamicin (100 μg/ml). Primary neuron cultures were incubated for 5 days in a humidified incubator at 37°C in an atmosphere of 5% CO_2 and 95% air.

Immunohistochemistry was performed. Cell cultures were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 2 hours at room temperature or 6 hours at 4°C. Fixed cultures were washed in phosphate buffer for 15 min at least three times and incubated in a 10% solution of bovine serum for at least 6 hours at 4°C. Cultures were incubated for at least 24 hours with primary antibodies to the astrocytic marker GFAP and neuronal marker NeuN (1:1000). Cultures were washed in phosphate buffer containing 0.1% bovine serum and 0.1% TritonX detergent. Cultures were incubated with secondary antibodies conjugated with fluorescent markers for at least 6 hours (1:1000). Cultures were washed in phosphate buffer for 15 minutes at least three times, stained with DAPI, and examined under an LSM 760 laser scanning confocal microscope (Zeiss, Germany).

Figures 4-5 show astrocytes and neurons transfected in vitro with genetic constructs (green) and immunohistochemically stained for the astrocyte-specific marker GFAP (red) and the neuron-specific marker NeuN (red).

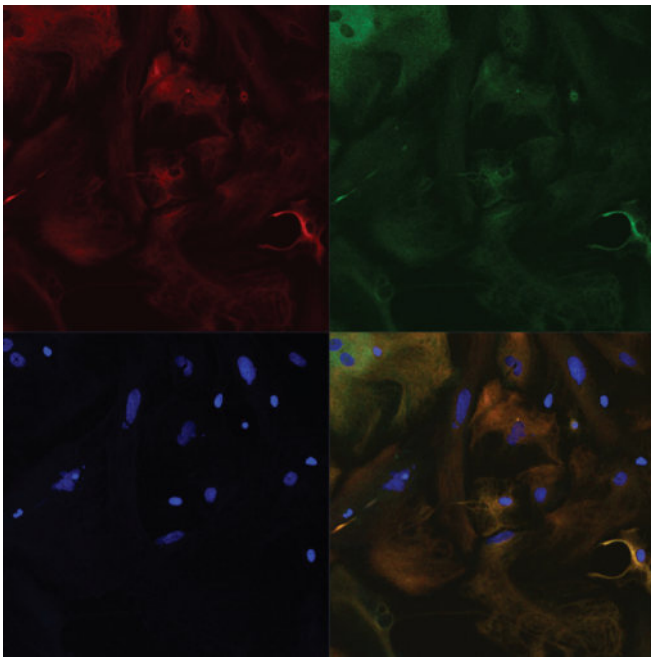


Figure 4. Astrocytes transfected with LVV-GFAP-GCaMP3 (green) and stained for the astrocyte-specific marker GFAP (red), in vitro

Colocalization of fluorescent signals produced yellow coloration at the cross-section, which allowed for the

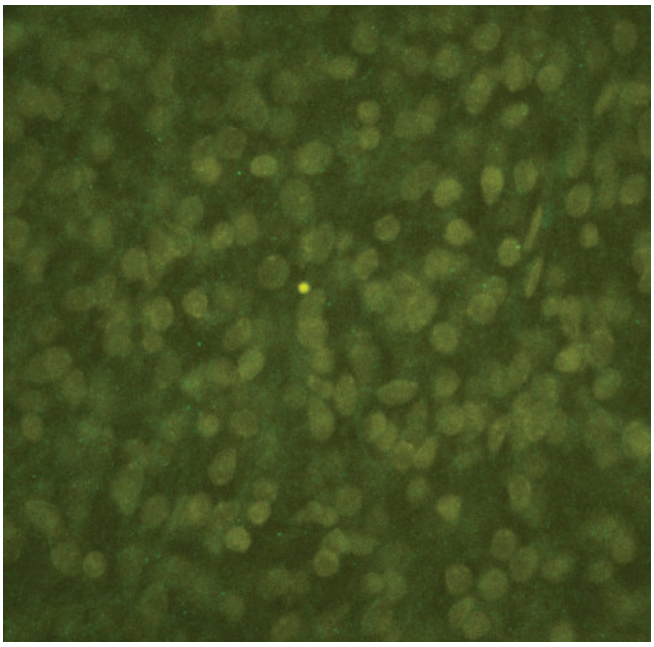


Figure 5. Neurons transfected with LVV-GFAP-TN-XXL (green) and stained for the neuron-specific marker NeuN, in vitro

quantification of cells stained only for GFAP or NeuN and cells with colocalized fluorescent signals. The specificity of the genetic construct to a particular cell type was calculated based on the number of cells per brain section that contained colocalized fluorescent signals.

The number of induced signalling cascades during SRMN-1 and SRMN-2 transfection was determined via activation of intracellular signalling cascades of neurons and astrocytes and recording of changes in fluorescence signal levels. Induced cascades were purine (purinergic) receptors P2X and P2Y, and β-adrenergic receptors. Therefore, three ingsignalling cascades were activated: X- and Y-type purine receptors and β-adrenergic receptors.

Substance-activator was added to induce signal cascades of SRMN-1 and SRMN-2 transfection in pure astrocyte and neuron cultures, and the concentration of intracellular Sa^{2+} was measured using fluorescence. Fluorescence levels were compared to levels prior to addition of the activator. Transfected cells that exhibited a two-fold or greater increase in fluorescence intensity after addition of the appropriate activator were considered activated.

Neurons and astrocytes were grown on cover glasses and transferred with genetic constructs for expression of LVV-PRSx8-TN-XXL and LVV-GFAP-GCaMP3, respectively. Growth media Neurobasal-A (neurons) and DMEM (astrocytes) was removed using fully balanced Hanks solution (HBSS) at room temperature, and cells were transferred to the experimental cell. Cells were undisturbed for 30 minutes at room temperature. The temperature of the HBSS solution was increased to 37°C immediately before the experiment, and the experimental cells were transferred to the specimen stage of a laser scanning confocal microscope. Fluorescence excitation of lasers and light filters were adjusted to record fluorescence (argon laser 488 nm, intensity 1%; 510–525 nm). The primary method of imaging activation of purinergic receptors was intracellular Ca^{2+} concentration. The following activators were used in pure cultures of astrocytes and neurons: 10 μM of 2-MeSATP was added to activate P2Y-dependent Ca^{2+} cascades; 10 μM a,b-mATP was used to activate P2X-dependent Ca^{2+} cascades; and 50 μM of isoproterenol was used to activate β–adrenergic-dependent Ca^{2+} cascades. Changes in fluorescence were recorded using an LSM 780 confocal microscope (Carl Zeiss).

The experimental design of SRMN sample efficiency **in vivo** included stereotactic injections into rat brains, post-surgical monitoring, preparation of brain slices expressing SRMN areas, and an assessment of transfection efficiency of SRMN using laser scanning confocal microscopy. The same methods described above (in vitro studies) were used to define the specificity and number of induced cascades during SRMN-1 and SRMN-2 transfection in vivo (Fig. 6).

Specificity for a certain cell type of each genetic construct was determined using in vivo transfection of rat brain cells, obtaining brain slices, followed by staining using immunohistochemistry for cell-specific markers (same antibodies as in vitro), and recording of fluorescent signals using an LSM 780 confocal microscope (Carl Zeiss).

Viral particles were injected into the brain according to the following protocol. The solution for brain preparation contained (in mM) NaCl 87, KCl 2.5, $MgSO_4$ 8.48, NaH_2PO_4 1.24, $NaHCO_3$ 26.2, $CaCl_2$ 0.5, and D-glucose 11, osmolarity (289-296 mmol/kg) and pH (7.4). The solution for slice incubation contained (in mM) NaCl 119; KCl, 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1; $NaHCO_3$ 26.2; $CaCl_2$ 1; $MgCl_2$ to 1.6; D-glucose 11; osmolarity (289-296 mmol/kg) and pH (7.4). The working Ringer's solution contained (in mM): NaCl 119; KCl, 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1; $NaHCO_3$ 26.2; $CaCl_2$ 2; D-glucose 11 (pH 7.4; osmolarity 295 mOsm). The rat was anaesthetized and fixed in a stereotaxic frame. A cannula hole was opened, and the needle of the adapter of the microdoser was inserted into the hole.. The solution was injected (GKKI lentiviral genetic structures – LVV-PRSx8-TN-XXL and LVV-GFAP-GCaMP3). Viral particles were introduced slowly at a rate not greater than 10 μl within 1 minute. The needle was removed from the cannula, and the hole was closed. Animals received post-surgical care. Rats were decapitated after the recovery period, and brains were isolated and immediately transferred to the preparation solution (4°C). Slices (40- to 50-μm thick) were prepared. Slices were taken from the bath of the vibratome using a brush and placed in carbogenized solution for incubation at 36°C. Slices were placed under a Zeiss 780 confocal microscope (Zeiss, Germany). Brain slices in Ringer's solution were placed in a chamber with specific gas and temperature conditions, which were adjusted using Zen software (Zeiss, Germany). Sharp slices were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.4) for 2 hours at room temperature. Subsequent processing was the same as the in vitro studies. Processing ended with laser scanning confocal microscopy.

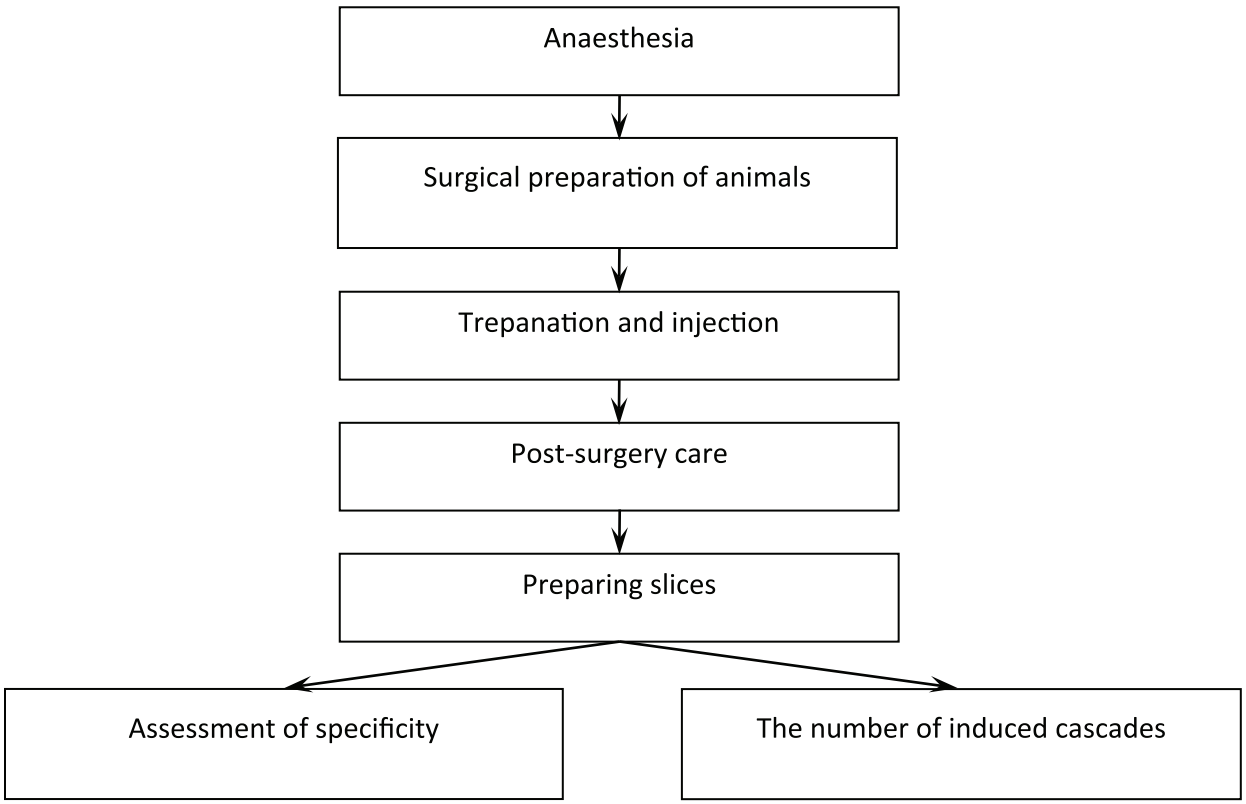


Figure 6. The plan of studying experimental SRMN samples for assessing their efficiency in the in vivo experiments

Results of the *in vitro* studies

Cells stained only for GFAP (astrocytes) or NeuN (neurons) and cells with colocalized fluorescent signals were counted. All genetic constructs withstood the experimental processing because fluorescent signals were registered from neurons and astrocytes transfected with a certain construct. The specificity of the genetic constructs for a certain cell type was greater than 97% for LVV-PRSx8-TN-XXL and LVV-GFAP-GCaMP3 (Table 1). Notably, the number of astrocytes was higher on average than the number of neurons.

The dynamics of calcium activity in astrocytes (Fig. 7) and neurons (Fig. 8) transfected in vitro with lentiviral vector LVV-GFAP-GCaMP3 and LVV-PRSx8-TN-XXL, respectively, in response to activation of P2X, P2Y and β-adrenergic dependent Ca^{2+} cascades were recorded.

All figures show the fluorescence intensity of the transfected brain cells cultures before activation of Ca^{2+}-dependent cascades and the fluorescence intensity and the dynamics of changes after activation of these cascades. The figures show samples SRMN No. 1.2 and 2.2, and similar data were obtained for other SRMN samples.

Table 1. The results of the in vitro research with definition of the specificity of the genetic constructs to a specific type of cell

SRMN sample	Type of transfected cell **in vitro**	Average number of cells (5 culture cups and slices)			Specificity of the genetic construct
		Number of cells immune-reactive for cell-specific marker	Number of cells successfully transfected with the appropriate genetic design	Number of cells immune-reactive for cell-specific marker and successfully transfected with a genetic construct	
No. 1.1	astrocytes	415±47	419±18	404±26	97.4%
No. 1.2		414±45	420±16	405±20	97.1%
No. 1.3		410±27	425±19	400±16	97.5%
No. 2.1	neurons	340±15	348±34	331±16	97.4%
No. 2.2		329 ±17	337 ±34	320 ±10	97.2%
No. 2.3		334 ±15	343 ±34	325 ±16	97.3%
Experimental data about the dynamics of cell activity are shown as values of fluorescence intensity in relative units.					

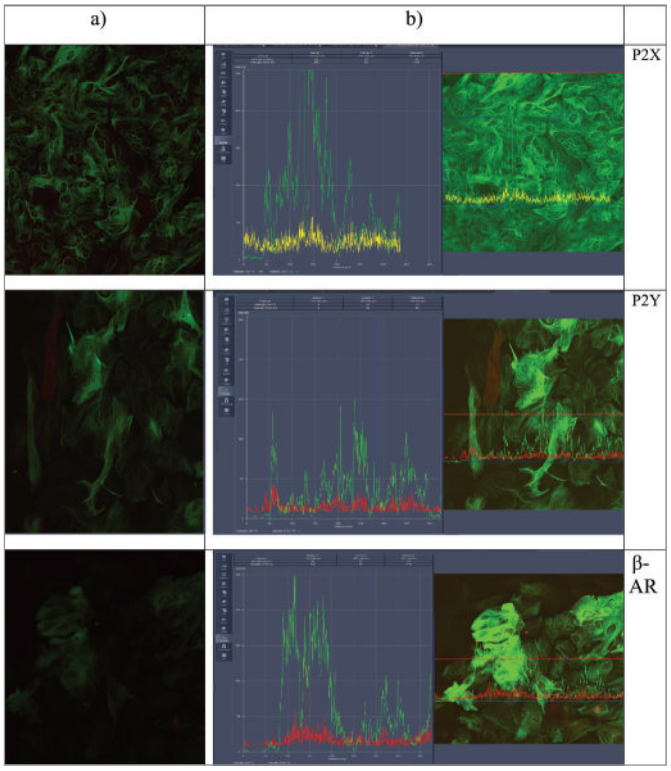


Figure 7. A pure culture of rat brain astroglia cells: a) astrocytes transfected with lentiviral vector LVV-GFAP-GCaMP3 in vitro and expressing GKKI G-CaMP3; b) astrocytes expressing GKKI G-CaMP3 when activated with P2X dependent CA2+ cascades (top), when activated with P2Y dependent Ca2+ cascades (middle), β–adrenergic dependent Ca2+ cascades (bottom).

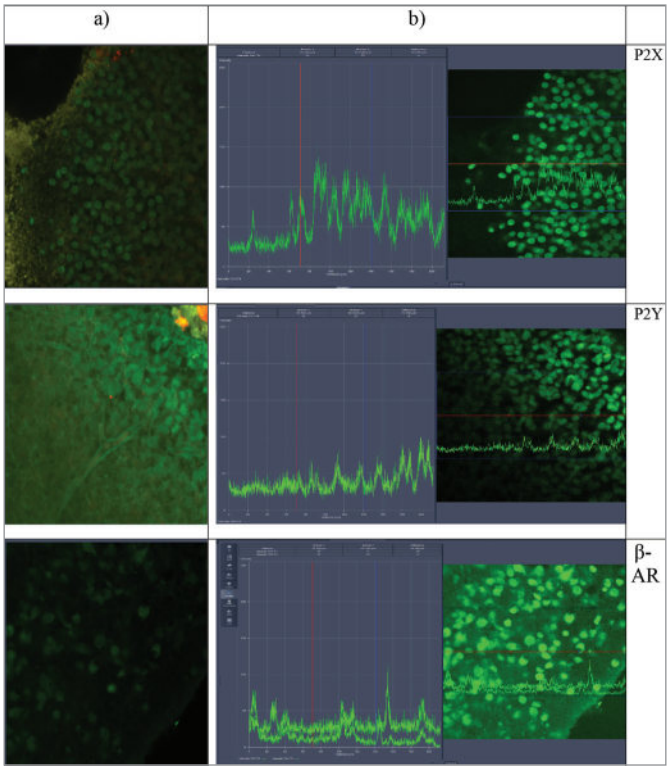


Figure 8. Pure culture of rat brain cells: a) neurons transfected with lentiviral vector LVV-PRSx8-TN-XXL in vitro and expressing GKKI TN-XXL; b) neurons expressing GKKI TN-XXL when activated with P2Y dependent CA2+ cascades (top), activated with P2Y-dependent Ca2+ cascades (middle), β–adrenergic dependent Ca2+ cascades (bottom).

Changes in the fluorescence intensity upwards (more than twice) confirmed the activation of intracellular signalling cascades in neurons and astrocytes. The increase in the fluorescence intensity in astrocytes was slightly higher than neurons.

Results of the *in vivo* studies

Figures 9-10 show astrocytes and neurons transfected in vivo with genetic constructs (green) and immunohistochemically stained for the astrocyte-specific marker GFAP

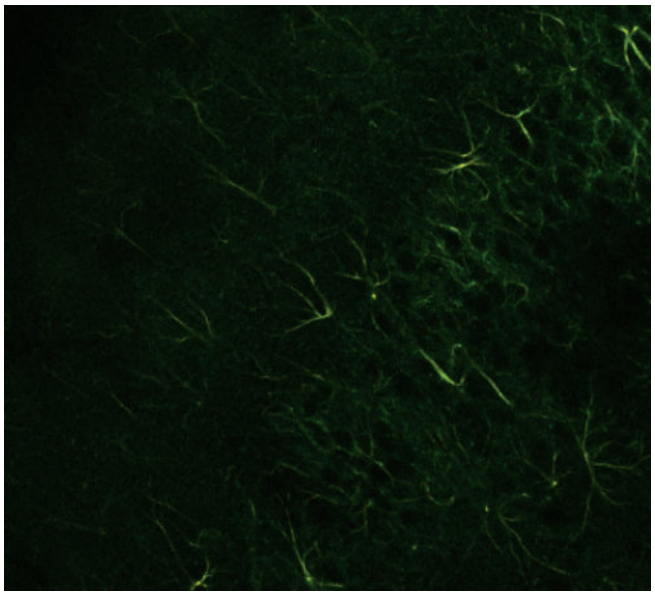


Figure 9. Astrocytes transfected with LVV-GFAP-GCaMP3 (green) and stained for the astrocyte-specific marker GFAP (red), in vivo

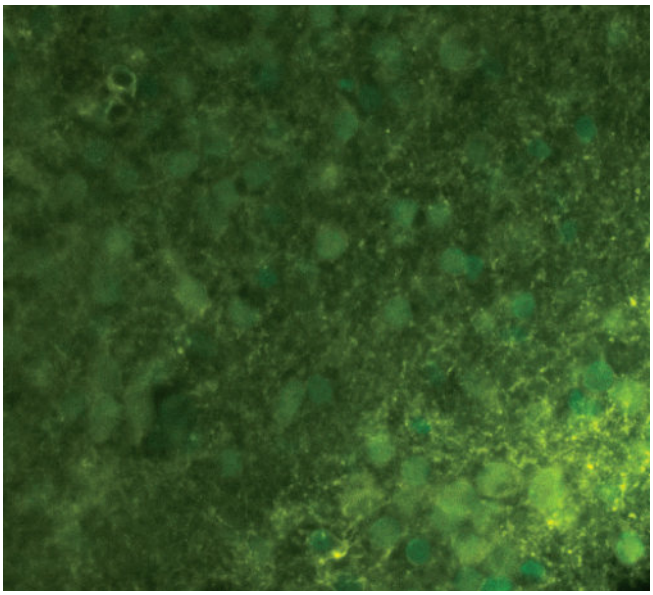


Figure 10. Neurons transfected with LVV-GFAP-TN-XXL (green) and stained for the neuron-specific marker NeuN (red), in vivo

Table 2. The results of the in vivo research with definition of the specificity of the genetic constructs to a specific type of cell

SRMN sample	Type of transfected cell	Number of cells immune-reactive for cell-specific marker	Number of cells successfully transfected with the appropriate genetic design	Number of cells immune-reactive for cell-specific marker and successfully transfected with a genetic construct	Specificity of the genetic construct
No. 1.1	astrocytes	410±34	408±18	422±16	97.2%
No. 1.2		400±25	410±20	388±14	97.0%
No. 1.3		415±47	414±22	404±26	97.3%
No. 2.1	neurons	330±16	341±34	321±16	97.3%
No. 2.2		340±15	365±42	330±16	97.1%
No. 2.3		323±10	340±37	314±16	97.2%

(red) and the neuron-specific marker NeuN (red). Colocalization of fluorescent signals produced a yellow coloration in the section.

Cells stained only for GFAP (astrocytes) or NeuN (neurons) and cells with colocalized fluorescent signals were counted (Table 2). All genetic constructs withstood the experimental processing because fluorescent signals were registered from neurons and astrocytes transfected with a certain construct. The specificity of genetic constructs for a certain cell type was calculated based on the number of cells in a slice of brain with colocalized fluorescent signal that was just over 97% for all samples.

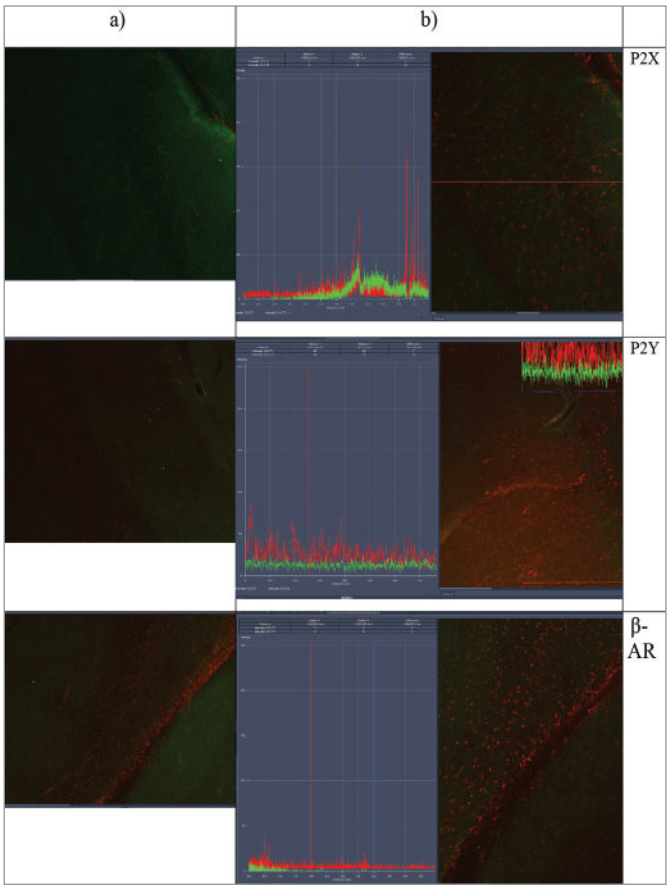


Figure 11. Organotypic rat brain slices: a) astrocytes transfected with lentiviral vector LVV-GFAP-GCaMP3 in vivo and expressing GKKI G-CaMP3; b) astrocytes expressing GKKI G-CaMP3 upon activation of P2X dependent Ca_2^+ cascades (top), upon activation of P2Y dependent Ca_2^+ cascades (middle), β – adrenergic dependent Ca_2^+cascades (bottom).

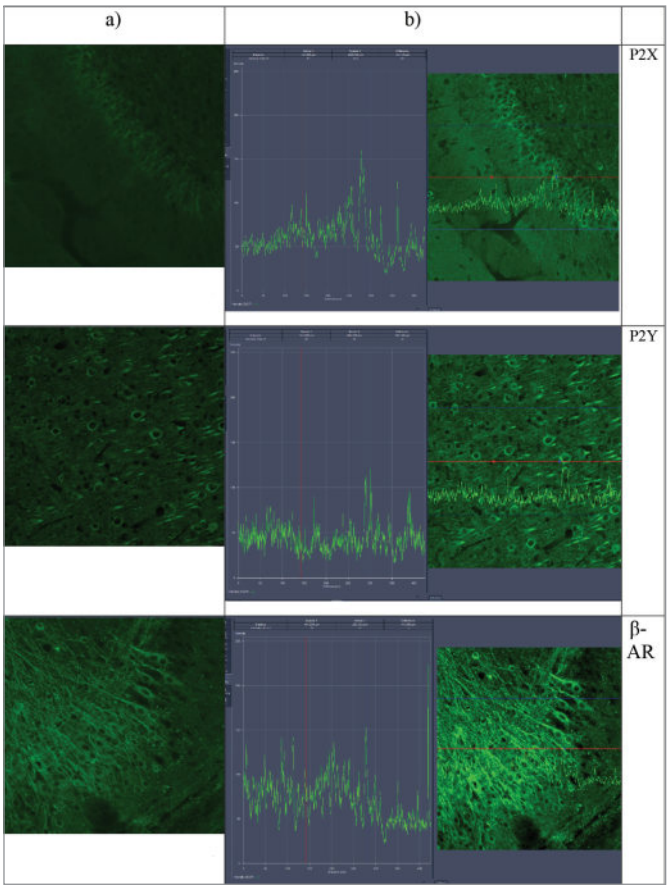


Figure 12. Organotypic rat brain slices: a) neurons transfected with lentiviral vector LVV-PRSx8-TN-XXL in vivo and expressing GKKI TN-XXL; b) neurons expressing GKKI TN-XXL upon activation of P2Y dependent Ca_2^+ cascades (top), upon activation of P2Y dependent Ca_2^+ cascades (middle), β – adrenergic dependent Ca2+cascades (bottom).

The results of the *in vivo* experiment repeated the in vitro experiment.

The number of induced signalling cascades in SRMN-1 and SRMN-2 transfection was determined similar to the in vitro studies. The only exception was that all experiments were performed on the sharp brain slices prepared in the same manner as determinations of the specificity of the genetic structures in vivo.

The dynamics of calcium activity in astrocytes (Fig. 11) and neurons (Fig. 12) transfected with appropriate lentiviral vector in vitro in response to activation of purinergic (P2X, P2Y) and β-dependent adrenal Ca^{2+} cascades exhibited comparable results to the in vitro experiments. All figures show the fluorescence intensity of transfected organotypical brain cells slices before activation of cascades and the fluorescence intensity and the dynamics of changes after activation of these Ca^{2+}-dependent cascades. All SRMN samples produced the same results.

The results revealed a significant upward change in the fluorescence intensity, which also confirmed activation of the intracellular signalling cascades in neurons and astrocytes.

DISCUSSION

Astrocytes were discovered and described approximately 150 years ago, and these cells are increasingly the subject of study in various areas. Adequate methods of detecting cells of the nervous system must be developed to implement fundamental discoveries, which was the goal of the authors of this study. The end products of the developed methods are sets of reactants for modelling neurogenesis (SRMN) containing genetic constructs based on the lentiviral vector with built-in cell-specific sequences that provide expression of these genetic structures in astrocytes (SRMN-1) or neurons (SRMN-2) in DMEM with 10% foetal bovine serum. The genetic construct successfully integrates into the genome of target cells to induce stable expression of the genetically encoded calcium indicator GCaMP3 for 48–72 hours after addition to cultures in vitro or after injection into the brain parenchyma in vivo.

The in vitro and in vivo experiments demonstrated a greater than two-fold increase in fluorescent signals in response to the addition of activators that stimulate purinergic and beta-adrenal calcium-dependent signalling cascades in various cells of the central nervous system and high specificity to a particular cell type (97%). These results confirmed the efficiency of the developed method. The results obtained in pure cell cultures were identical to the results in animals.

An important fundamental conclusion of this work is evidence of the critical role of astrocytes in ensuring neurogenesis and neuroplasticity.

The developed method of genetic detection of neurogenesis is based on the use of a cellular-specific promoter to control the simultaneous expression of the required transgene and strong artificial activator of transcription to enhance transcription via binding to specific binding sites introduced into the sequence of the promoter. Therefore, two cell cycle-specific promoters are used: one for transcription of the transgene of interest, and the other for the expression of the transactivator.

The results of this method of assessing the efficiency of experimental SRMN samples revealed the successful and stable transfection of catecholaminergic neurons and astrocytes, experimental data on transfection efficiency, the specificity of the developed genetic structures as part of SRMN, and calcium dynamics in transfected neurons and astrocytes.

The use of this method allows cell- and phenotype-specific transfection of various types of neurons that ensures the targeted control of intracellular cascades (13) and visualization of in vitro-transfected cells via the embedding of genetically encoded calcium indicators into the developed genetic construct.

CONCLUSION

The developed methods and sets of reactants for modelling neurogenesis are intended to investigate brain functioning, particularly neural networks of mammalian brains using analyses of calcium dynamics in catecholaminergic neurons and astrocytes. These methods allow investigations of the functional status of brain cells in the normal state and in various pathologies. The scope of this methodology application is limited to fundamental and scientific research in neurosciences and molecular biology, particularly the molecular mechanisms of various diseases of the central nervous system. However, this development may become a routine method to monitor the functional state of neurons and astrocytes *in vivo* to examine developed medicines and monitor the efficiency of new methods for the treatment of schizophrenia, neurodegeneration, cerebrovascular, demyelination, and other highly disabling and socially significant diseases.

Notably, this technology based on cell viral transfection provides the possibility of non-invasive application, of which the best use is chemogenetic expression monitoring. This technique achieves specificity of expression of incorporated genes in certain brain areas. Therefore, our and similar developments may be introduced into clinical practice in the future to create a new type of gene therapy for diseases that cause degradation of neurons, astrocytes and other brain cells.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education and Science of the Russian Federation grant (FCP No. 14.575.21.0074 (unique identifier – RFMEFI57514X0074) in the Immanuel Kant Baltic Federal University.

REFERENCES

1. Erkkinen M.G., Kim M.O., Geschwind M.D. Clinical Neurology and Epidemiology of the Major Neurodegenerative Diseases. Cold Spring Harb Perspect Biol. 2017; (17): a033118.
2. Rumyantseva S.A., Silina E.V., Svischeva S.R., Komarov A.N. Medical and organizational issues of pre- and post-stroke disablement. Zhurnal Nevrologii i Psihiatrii imeni S.S. Korsakova. 2013;9:43-49.
3. Gomazkov O.A., Afanasiev V.V., Rumyantseva S.A., Stupin V.A., Silina E.V., Sokhova O.A. Current concepts of neurocytoprotective therapy. Neuroscience and Behavioral Physiology, 2013;3(43):374-379.
4. Verkhratsky A. Sofroniew M., Messing A. et al. Neurological diseases as primary gliopathies: a reassessment of neurocentrism. ASN Neuro. 2012;4:e00082. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3320215/
5. Khakh B.S., Sofroniew M.V. Diversity of astrocyte functions and phenotypes in neural circuits. Nat Neurosci. 2015;18:942–952.
6. Gray M.T., Woulfe J.M. Striatal blood-brain barrier permeability in Parkinson's disease. J. Cereb. Blood Flow Metab. 2015;35:747–750.
7. Herculano-Houzel S. The glia/neuron ratio: how it varies uniformly across brain structures and species and what that means for brain physiology and evolution. Glia. 2014;62:1377–1391.
8. Haydon PG, Nedergaard M. How do astrocytes participate in neural plasticity? Cold Spring Harb Perspect Biol. 2014;11;7(3):a020438.
9. Zhang Y., Sloan S. A., Clarke L. E., Caneda C., Plaza C. A., Blumenthal P. D., et al. Purification and characterization of progenitor and mature human astrocytes reveals transcriptional and functional differences with mouse. Neuron. 2016;89:37–53.
10. Sofroniew M.V., Vinters H.V. Astrocytes: biology and pathology. Acta Neuropathol. 2010;119:7–35.
11. Zuchero J. B., Barres B. A. Glia in mammalian development and disease. Development 2015;142:3805–3809.
12. Booth H., Hirst W., Wade-Martins R. The Role of Astrocyte Dysfunction in Parkinson's Disease Pathogenesis. Trends Neurosci. 2017; 40(6): 358–370.
13. Kasymov V.A., Bogdanov E.A., Silina E.V., Litvitsky P.F., Shusharina N.N., Patrushev M.V. Evaluation of the functional status of signal systems of astrocytes by the developed reagent kit basedon genetic constructions (experimental study). Asian Journal of Pharmaceutics. 2017;11 (4):787-793.

ESOPHAGEAL MOBILIZATION IN THE TREATMENT OF SHORT ESOPHAGUS

Dragan Canovic[1,2], Bojan Milosevic[1,2], Dejan Lazic[1,2], Aleksandar Cvetkovic[1,2], Marko Spasic[1,2], Bojan Stojanovic[1,2], Slobodanka Mitrovic[2,3], Mladen Pavlovic[1,2]

[1] Clinic for General and Thoracic surgery, Clinical center Kragujevac, Kragujevac, Serbia
[2] Faculty of Medical Sciences, University of Kragujevac, Kragujevac, Serbia
[3] Department for pathologic and anatomic diagnostics, Clinical center Kragujevac, Kragujevac, Serbia

MOBILIZACIJA JEDNJAKA U LEČENJU KRATKOG JEDNJAKA

Dragan Canović[1,2], Bojan Milosević[1,2], Dejan Lazić[1,2], Aleksandar Cvetković[1,2], Marko Spasić[1,2], Bojan Stojanović[1,2], Slobodanka Mitrović[2,3], Mladen Pavlović[1,2]

[1]Klinika za opštu i grudnu hirurgiju, Klinički centar Kragujevac, Kragujevac, Srbija
[2]Fakultet medicinskih nauka, Univerzitet u Kragujevcu, Kragujevac, Srbija
[3] Služba za patološku anatomsku dijagnostiku, Klinički centar Kragujevac, Kragujevac, Srbija

Received / Primljen: 15. 10. 2016. Accepted / Prihvaćen: 25. 10. 2016.

ABSTRACT

Short esophagus is well known complication of a long term gastroesophageal disease. There are several ways to solve this problem intraoperatively. One of the first steps is extensive esophageal mobilisation. In this review we emphasize different approaches and types of this procedure, with their advantages and disadvantages.

Keywords: *Esophagus, short; frenotomy; vagotomy*

SAŽETAK

Sindrom kratkog jednjaka je dobro poznata komplikacija dugotrajne gastroezofagealne refluksne bolesti. Postoji nekoliko načina za rešavanje ovog problema intraoperativno. Jedan od prvih koraka je ekstenzivna mobilizacija jednjaka. U ovom radu dajemo pregled različitih pristupa i vrsta ove procedure, sa svojim prednostima i manama.

Ključne reči: *Kratak jednjak; frenotomija; vagotomija*

INTRODUCTION

The short esophagus (SE) is a recognized complication of long-standing gastroesophageal reflux disease (GERD) wherein the gastroesophageal junction (GEJ) cannot be sufficiently reduced below the crura of the diaphragm (1). For many years this syndrome creates controversy among surgeons. Although some even claim that this entity does not exist, there are many esophageal surgeons who agree that long-term reflux disease, scarring and axial contraction of the longitudinal muscle layer of the esophagus leads to its shortening (2,3). The only way to confirm the presence of a short esophagus is intraoperative inability to maintain the gastroesophageal junction below the diaphragm without significant tension (4–7). A fundoplication performed around an intrinsically short esophagus without a lengthening procedure will have a high failure rate because of mediastinal wrap herniation or disruption due to excessive tension. This is believed to be responsible for approximately 20–35% of the surgical failures after open or laparoscopic antireflux surgery (8–10). In the era of laparoscopic antireflux surgery it is imperative to identify the short esophagus preoperatively. Although many of diagnostic procedures were used to assess abdominal esophageal length preoperatively, no one had a significant sensitivity and specificity. Some risk factors were used earlier as preopearative predictors of this syndrome, with low sensitivity and specificity, such as irreducible hiatus hernia greater than 5 cm, paraesophageal hernia, stricture/fibrosis, Barrett`s esophagus and short manometric length (3,4,6). Yano et al established esophageal length index (ELI) and endoscopic esophageal length (EEL) as a valuable additional predictors of short esophagus (11,12). These parameters had significantly higher sensitivity and specificity for this final stage of GERD.

Key part in reflux disease treating is successfully launching esophagogastric junction below the diaphragm with a minimum tension, for at least 2.5 cm below the esophageal hiatus, which is the acceptable length that guarantees antireflux valve positioning in the abdomen and its functioning (8,13). The most common diseases in which mobilization of the esophagus is carried out, near the syndrome of the short esophagus, are malignant diseases of the esophagogastric junction and corrosive damage to the esophagus. Achieving a technically satisfactory reconstruction of the cardia by way of an antireflux procedure is substantially more difficult under these conditions. Even when a good

sciendo

UDK: 616.329-089; 616.333-008.8-06 / Ser J Exp Clin Res 2018; 19 (2): 185-188
DOI: 10.1515/SJECR-2016-0086

Corresponding author: Bojan Milosevic
Zmaj Jovina Street, No30; Clinic for General and Thoracic Surgery, Clinical center Kragujevac
Email address: bojanzmilosevic@gmail.com; Phone number +38163625686

repair may be achieved, its durability may be compromised by the continued cephalad tension on the gastroesophageal junction, which may lead to recurrent herniation. Surgicaly lengthening of the short esophagus can be achieved in several ways. First is extensive mediastinal mobilization and division of the esophagus from the mediastinal structures. Second, when this method is not enough, is to perform a Collis gastroplasty. Third and somewhat extreme solution is to perform an esophageal resection. There are several approaches to perform mediastinal mobilization for obtaining adequate esophageal length. In this paper we review these methods.

Types of mobilization

There are two main types of esophageal mobilization (14). Type 1 is standard for fundoplication, and consists of complete dissection and exposure of diaphragmatic pillars, specially of the right pillar, by means of the opening of the hepatoduodenal and hepatogastric ligaments, side by side with the release of the gastric end of the diaphragm (15,16). At this step, care is taken to preserve the hepatic branch of the vagus nerve. Also, the peritoneum and the phrenoesophageal membrane should be lifted, thus mobilizing the esophagus in the posterior mediastinum and keeping the baseline morphology of the esophageal hiatus. Type 2 implies transhiatal large mediastinal dissection, with separation of the esophageal body from surrounding mediastinal structures (14).

Procedure approaches

Two approaches that most common have been used for the mobilization of the esophagus are transabdominal and transthoracic. Within them are considered laparotomy and thoracotomy, and lately laparoscopic and thoracoscopic approach. These approaches are the subject of many studies that seek to determine whether there is an advantage over others in terms of providing an adequate length of intra-abdominal part of the esophagus with minimal post-operative complications (14,17). As an alternative method mentioned Collis gastroplasty which can be performed via laparotomy and by laparoscopy. This technique consists in increasing the length of the abdominal part of the esophagus through gastroplasty conducted in parallel with the axis of the esophagus (18–20). This method has been criticized because of the presence of parietal cells that secrete gastric acid in neoesophagus and because of reduced motility that allows direct contact between the acidic contents and mucosa of the esophagus (1,11,19). Despite this gastroplasty and fundoplication can be effective in controlling symptoms of the reflux (10,17). Traditionally, in patients with suspected syndrome of short esophagus is conducted transthoracic fundoplication because this approach provides ideal conditions for the mobilization of the esophagus and allows Collis gastroplasty when it is needed.

Frenotomy

Laparotomy approach gives good results, although it has been shown that a wide frenotomy can be very important in the implementation of mediastinal dissection, which increases the length of the abdominal part of the esophagus for about 1 cm as compared to patients who never opened diaphragm (the average length of the esophagus obtained during frenotomy is 3.24 cm compared to 2.12 cm in patients with laparotomy only) (14,21). These results indicate that frenotomy, although it is not a routine procedure, can be an alternative approach when, during laparotomy approach, is established that antireflux valve is not able to stay in the abdomen.

Vagotomy

Left anterolateral thoracotomy is approach through which is possible to do a dissection of the esophagus and afterwards anterior and posterior vagotomy (the length of the esophagus, which is obtained after anterior vagotomy is approximately 1.12 centimeters while after the posterior vagotomy is about 0.97 cm). In this way the length of the esophagus from about 3.81 cm can be achieved, which is significantly more than by laparotomy (21). Lately described a new technique of vagus nerve division that should make a greater length of the esophagus. It consists in the dissection of the both vagus nerves at three levels, resulting in an increase of the length of the abdominal esophagus (3.7 cm ± 1.2 cm) (22). The key technical moments are blunt distal esophagus preparation of its intrathoracic attachment which includes regional lymphatics of the lower mediastinum and multiple vagal transections at several levels. Number of vagus nerve transections (three) is necessary because, after transection at each level about 1 cm lengthening of the esophagus is achieved. The final length represents the additive effect of the individual transections. Although the complete dissociation of the esophagus from the nerves is technically feasible, it should be avoided because of the risk of extensive laceration of muscular layer and even perforation. This approach is not entirely accepted among surgeons. In a certain number of patients who are operated for peptic ulcer occurs the disorder in the motility of the stomach, which often require additional drainage procedure. Role of this procedure in the treatment of short esophagus was examined in a prospective study and it was found that does not lead to increased rates of delayed gastric emptying nor other possible effects (21).

Laparoscopy approach

Introduction of laparoscopic antireflux surgery and the development of techniques that allow laparoscopic mediastinal mobilization of the esophagus, raise the question whether it is possible to get a similar length of the esophagus with less invasive laparoscopic approach.

In this regard conducted experimental studies on animals (pigs) where laparoscopic and thoracoscopic mobilization have been done, as well as mobilization with or without bilateral section of the vagus nerve. Laparoscopic mobilization average increase in length of the esophagus was only 4mm, which is significantly less than the average increase in length of 12 mm with transthoracic esophageal mobilization. The average length of next 6 mm was obtained with transthoracic access after laparoscopic mobilization (23). Further increasing the length of the esophagus was obtained with section of the vagus nerves, but there were no a significant difference of the length with division of the anterior in relation to the posterior vagus nerve obtained. The maximum mobilization of the esophagus, an average of 18.5 mm was obtained by a complete esophageal mobilization and bilateral section of vagi. It is shown that transthoracic mobilization leads to a significant increase in the length of the esophagus compared to laparoscopic mobilization (24). Laparoscopic visualization of the esophagus in the lower mediastinum is an excellent but the dissection of the esophagus below the lung hilum does not provide a large increase in the length of the esophagus. It was established that during transthoracic mobilization part of laparoscopic dissection who did not satisfactorily done is the preparation of the bronchial arteries and branches of the vagus to the hilus, especially in main bronchi. It would be possible to implement a similar preparation laparoscopically but work high in the mediastinum, near the pulmonary artery and bronchial blood vessels is a difficult and potentially dangerous (25). Although, minimal mobilization of the esophagus during laparoscopic fundoplication reduces postoperative migration and the need for the reoperation. It was established that dissection of the vagus nerve without esophageal mobilization significantly increases the length of the esophagus (21). However the maximum length of the esophagus can be achieved by a combination of mediastinal mobilization and vagotomy. Even when the main vagus is dissected, small branches to the lung hilus make difficult to obtain the desired length of the esophagus. Section of small branches of the vagus does not eliminate the fixating role of this nerve. This indicates that in the case of mobilization of the esophagus itself is not sufficient to ensure the maintenance of esophagogastric junction below the diaphragm, one should take into account the vagus dissection (26,27). Unilateral vagal injuries is relatively common, especially in antireflux surgery procedures, and usually are well tolerated. Transection of both major vagus branches will give an even greater increase in the length of the esophagus, but morbidity due to bilateral truncal vagotomy can be greater than the benefits of additional length of the esophagus, especially when you take into account the possibility of Collis gastroplasty (21,28). Due to the short length of the esophagus which is obtained by unilateral vagotomy this technique should not be used as a replacement for the mobilization of the esophagus. Extensive esophageal mobilization alone should be used as the primary method to increase the esophageal length. Since thoracotomy can be a significant cause of respiratory complications, endoscopic methods for mobilization, resection and reconstruction of the esophagus have been widely used. During thoracoscopic mobilization right lung deflation is done, and in about one-quarter of patients is necessary to apply antibiotic treatment for respiratory problems. A small number of previous studies have shown that there is no advantage of the thoracoscopic mobilization compared to the open approach, but some studies showed that the thoracoscopic approach does have a larger number of complications compared to traditional, open technique, although in this study mobilization of the esophagus as a component part of esophagectomy was examined (14,25,29,30). Based on previous studies it was shown that the maximum length of the abdominal part of the esophagus was obtained using thoracic approach (3). However, given that in this approach bilateral vagotomy is a condition for obtaining the maximum length of the esophagus, which carries a certain morbidity and postoperative complications, laparotomy approach should be used and Collis gastroplasty if necessary.

CONCLUSION

This common problem of short esophagus requires high volume centers and experienced surgeons to solve. There are several approaches to establish enough abdominal esophageal length, and they depend on type of the disease that caused axial esophageal shortening. Esophageal mobilization is often enough to achieve enough esophageal length, either performed through thoracic or laparotomy approach. Frenotomy or vagotomy, in combination with minimally invasive approaches, can be additional maneuvers that help to avoid gastroplasty procedures.

REFERENCES

1. Nason KS, Luketich JD, Awais O, Abbas G, Pennathur A, Landreneau RJ, et al. Quality of life after collis gastroplasty for short esophagus in patients with paraesophageal hernia. Ann Thorac Surg. 2011 Nov;92(5):1851–4.
2. Mattioli S, Lugaresi ML. Lengthening Gastroplasty for Managing Gastroesophageal Reflux Disease and Stricture. In: Ferguson MK, editor. Difficult Decisions in Thoracic Surgery: An Evidence-Based Approach. London: Springer London; 2007. p. 305–17.
3. Teitelbaum EN, Soper NJ. The Short Esophagus. In: Swanstrom LL, Dunst MC, editors. Antireflux Surgery. New York, NY: Springer New York; 2015. p. 177–87.
4. Gastal OL, Hagen JA, Peters JH, Campos GM, Hashemi M, Theisen J, et al. Short esophagus: analysis of predictors and clinical implications. Arch Surg. 1999 Jun;134(6):633–8.

5. Bochkarev V, Lee YK, Vitamvas M, Oleynikov D. Short esophagus: how much length can we get? Surg Endosc. 2008 Oct;22(10):2123–7.
6. Low DE. The short esophagus recognition and management. J Gastrointest Surg. 2001;5(5):458–61.
7. Herbella FAM, Del Grande JC, Colleoni R. Short esophagus: literature incidence. Dis Esophagus. 2002;15(2):125–31.
8. Jobe BA, Richter JE, Hoppo T, Peters JH, Bell R, Dengler WC, et al. Preoperative Diagnostic Workup before Antireflux Surgery: An Evidence and Experience-Based Consensus of the Esophageal Diagnostic Advisory Panel. J Am Coll Surg. 2016 Sep 18;217(4):586–97.
9. Horvath KD, Swanstrom LL, Jobe BA. The short esophagus: pathophysiology, incidence, presentation, and treatment in the era of laparoscopic antireflux surgery. Ann Surg. 2000 Nov;232(5):630–40.
10. Zehetner J, DeMeester SR, Ayazi S, Kilday P, Alicuben ET, DeMeester TR. Laparoscopic Wedge Fundectomy for Collis Gastroplasty Creation in Patients With a Foreshortened Esophagus. Ann Surg. 2014;260(6).
11. Garg N, Yano F, Filipi CJ, Mittal SK. Long-term symptomatic outcomes after Collis gastroplasty with fundoplication. Dis Esophagus. 2009;22(6):532–8.
12. Yano F, Stadlhuber RJ, Tsuboi K, Garg N, Filipi CJ, Mittal SK. Preoperative predictability of the short esophagus: endoscopic criteria. Surg Endosc. 2009 Jun;23(6):1308–12.
13. Herbella FAM, Del Grande JC, Colleoni R. Short esophagus or bad dissected esophagus? An experimental cadaveric study. J Gastrointest Surg. 2003;7(6):721–5.
14. Beduschi T, Bigolin AV, Cavazzola LT. Thoracotomy versus transhiatal esophageal dissection: which is the best surgical approach to short esophagus? Acta Cir Bras. 2011;26(3):214–9.
15. St. Peter SD, Barnhart DC, Ostlie DJ, Tsao K, Leys CM, Sharp SW, et al. Minimal vs extensive esophageal mobilization during laparoscopic fundoplication: A prospective randomized trial. J Pediatr Surg. 2011;46(1):163–7.
16. Lee JW, Sung SW, Park JK, Park CH, Song KY. Laparoscopic gastric tube formation with pyloromyotomy for reconstruction in patients with esophageal cancer. Ann Surg Treat Res. 2015;89(3):117–23.
17. Durand L, De Anton R, Caracoche M, Covian E, Gimenez M, Ferraina P, et al. Short esophagus: selection of patients for surgery and long-term results. Surg Endosc. 2012 Mar;26(3):704–13.
18. Alaeddine MH, Safadi BY. Laparoscopic Collis Gastroplasty and Nissen Fundoplication. In: Hoballah JJ, Scott-Conner MD HCE, editors. Operative Dictations in General and Vascular Surgery. New York, NY: Springer New York; 2012. p. 50–4.
19. Mor A, Lutfi R, Torquati A. Esophageal acid-clearance physiology is altered after Nissen-Collis gastroplasty. Surg Endosc. 2013 Apr;27(4):1334–8.
20. Pera M, Deschamps C, Taillefer R, Duranceau A. Uncut Collis-Nissen gastroplasty: early functional results. Ann Thorac Surg. 1995 Oct;60(4):915–20; discussion 921.
21. Oelschlager BK, Yamamoto K, Woltman T, Pellegrini C. Vagotomy during hiatal hernia repair: A benign esophageal lengthening procedure. J Gastrointest Surg. 2008;12(7):1155–62.
22. Arkadopoulos N, Marinis A, Dafnios N, Theodosopoulos T, Smyrniotis V. A novel technique for gaining extra length of the abdominal esophagus. Am J Surg. 2009 Jul;198(1):e10-2.
23. Smithers BM, Gotley DC, McEwan D, Martin I, Bessell J, Doyle L. Thoracoscopic mobilization of the esophagus. Surg Endosc. 2001;15(2):176–82.
24. Worrell SG, Boys JA, DeMeester SR. Identification and Management of a ``Short Esophagus" and a Complex Hiatus. In: Aye WR, Hunter GJ, editors. Fundoplication Surgery: A Clinical Guide to Optimizing Results. Cham: Springer International Publishing; 2016. p. 31–7.
25. Ma Z, Niu H, Gong T. Thoracoscopic and laparoscopic radical esophagectomy with lateral-prone position. J Thorac Dis. 2014;6(2):156–60.
26. Tsuboi K, Omura N, Yano F, Hoshino M, Yamamoto SR, Akimoto S, et al. A novel laparoscopic approach for severe esophageal stenosis due to reflux esophagitis: how to do it. Surg Today. 2015 Feb;45(2):253–7.
27. Yau P, Watson DI, Jamieson GG, Myers J, Ascott N. The influence of esophageal length on outcomes after laparoscopic fundoplication. J Am Coll Surg. 2000 Oct;191(4):360–5.
28. Oelschlager B, Yamamoto K, Woltman T, Pellegrini C. Re: Oelschlager BK, Yamamoto K, Woltman T, Pellegrini C. Vagotomy during hiatal hernia repair: A benign esophageal lengthening procedure. J Gastrointest Surg. 2008;12(7):1155-62. J Gastrointest Surg. 2009;13(2):395.
29. Mattioli S. Difficult Decisions in Thoracic Surgery. Ferguson K M, editor. 2007.
30. Zilberstein B, Cecconello I, Pollara W, Domene CE, Nasi A, Pinotti HW. Esophagectomy Without Thoracotomy Using the Cervicoabdominal Approach for the Management of Esophageal Carcinoma: Results. In: Siewert JR, Hölscher AH, editors. Diseases of the Esophagus. Berlin, Heidelberg: Springer Berlin Heidelberg; 1988. p. 398–402.

APPLYING THE MOLECULAR ADSORBENT RECIRCULATING SYSTEM (MARS) IN THE TREATMENT OF ACUTE LIVER FAILURE (ALF) CASE REPORT

Jagoda Gavrilovic[1], Jelena Djordjevic Velickovic[1], Zeljko Mijailovic[1,2], Tatjana Lazarevic[2,3], Aleksandar Gavrilovic[2,4], Miroslav Tomovic[5,6]
[1]Department of infectious diseases of University Clinical center of Kragujevac, Serbia
[2]Faculty of Medical Sciences University of Kragujevac, Serbia
[3]Department of nephrology and dialysis, Clinical center Kragujevac, Serbia
[4]Department of neurology, Clinical center Kragujevac, Serbia
[5]Department of clinical chemistry and laboratory medicine of General Hospital of Petrovac, Serbia
[6]School of Medicine University of Belgrade, Belgrade, Serbia

PRIMENA MARS (eng MOLECULAR ADSORBENT RECIRCULATING SYSTEM) U LEČENJU AKUTNE INSUFICIJENCIJE JETRE (AIJ) PRIKAZ SLUČAJA

Jagoda Gavrilović[1], Jelena Đorđević Veličković[1], Željko Mijailović[1,2], Tatjana Lazarević[2,3], Aleksandar Gavrilović[2,4], Miroslav Tomović[5,6]
[1] Klinika za infektivne bolesti Klinički centar Kragujevac,Srbija
[2] Fakultet medicinskih nauka Univerzitet u Kragujevcu, Srbija
[3] Klinika za nefrologiju i dijalizu Klinički centar Kragujevac ,Srbija
[4] Klinika za neurologiju Klinički centar Kragujevac,Srbija
[5] Odsek kliničke biohemije, Opšta bolnica,Petrovac, Srbija
[6] Medicinski fakultet Univerziteta u Beogradu, Beograd, Srbija

Received / Primljen: 25. 07. 2016. Accepted / Prihvaćen: 07. 03. 2017.

ABSTRACT

Acute liver failure (ALF) is a rare but life-threatening illness with multiple organ failure. The short-term mortality rate exceeded 80 % despite modern approaches in treatment. Drugs, infections by hepatic viruses and toxins are the most common causes of ALF. Progressive jaundice, coagulation disorder and hepatic encephalopathy are dominated as a clinical signs of the illness. We present a case of a 36-year-old Caucasian woman hospitalized in ICU due to yellow discoloration of the skin and sclera, severe disseminated coagulopathy and hemodynamic instability. ALF is developed due to Hepatitis B Virus infection, resulting in hepatic toxicity as well as coma. General condition rapidly improved after applying of Molecular Adsorbent Recirculating System (MARS), an extracorporeal liver support system based on albumin dialysis. It is relatively expensive treatment that is used for the patient with hepatic encephalopathy grade 3 or 4 in our institution. In conclusion, an early administration of MARS significantly reveals subjective and objective clinical improvement in the case we presented.

Keywords: *Hepatitis B, acute viral; Encephalopathy, hepatic; Treatment, early; Liver dialysis; Survival;*

SAŽETAK

Akutna insuficijencija jetre (AIJ) je retka, ali po život opasna bolest sa multiplmom disfunkcijom organa. Kratkoročni mortalitet je preko 80% uprkos modernim pristupima u lečenju. Lekovi, infekcije hepatotropnim virusima i toksini su najčešći uzroci ALF. U kliničkoj slici dominiraju progresivna žutica, poremećaj koagulacije i hepatična encefalopatija. Predstavljamo slučaj 36-godišnje žene hospitalizovane u JIL zbog žute boje kože i sklera, teške diseminirane koagulopatije i hemodinamske nestabilnosti. ALF je nastala tokom infekcije virusom hepatitisa B, koja je rezultirala insuficijencijom jetre i komom. Opšte stanje se brzo poboljšalo nakon primene sistema za recirkulaciju molekularnih adsorbenata (MARS), ekstrakorporealnog sistema za podršku jetre na bazi albuminske dijalize. To je relativno skupo lečenje koje se koristi za pacijenta sa hepatičnom encefalopatijom stadijuma tri ili četiri. U zaključku, rana primena MARS-a rezultirala je značajnim subjektivnim i objektivnim kliničkim poboljšanjem u prikazanom slučaju.

Ključne reči: *Hepatitis B, akutni virusni; hepatička encefalopatija; lečenje, dijaliza jetre, preživljavanje*

ABBREVIATIONS

ABP – Arterial Blood Pressure
MAP – Mean Arterial Pressure
GCS – Glasgow Coma Score
APACHE II – Acute Physiology and Chronic Health Evaluation score II
ALF – Acute liver failure
ICU – Intensive Care Unit
MARS – Molecular Adsorbent Recirculating System
HE – Hepatic encephalopathy
ARDS – Acute Respiratory Distress Syndrome
SMT – Standard Medical Therapy
SOFA - Sequential Organ Failure Assessment score
MELD – Model for End-stage Liver Disease
ACT – Activated clotting time
INR – International Normalized Ratio
ALT – Alanine aminotransferase
AST – Aspartate aminotransferase
CVVHDF – Continuous venovenous hemodiafiltration
HRS – Hepatorenal syndrome
RRT - Renal Replacement Therapy
AFP – α (alpha)-fetoprotein
LPC - liver progenitor cells
MH – mature hepatocytes
IU/L – International Unit per liter

sciendo
UDK: 616.36-008.64-78 / Ser J Exp Clin Res 2018; 19 (2): 189-194
DOI: 10.1515/SJECR-2017-0074

Corresponding author: Miroslav S. Tomovic, MD,
The Department of clinical chemistry and laboratory medicine; General Hospital of Petrovac,
Mlavska Str # 18, 12300 Petrovac, Republic of Serbia, Tel: +381604031979; E-mail: mocakg@open.telekom.rs

INTRODUCTION

Acute liver failure (ALF) is a rare but life-threatening critical illness that occurs most often in patients who do not have preexisting liver disease (1). The predominant causes are acute viral infections (hepatitis A, B and E), acute alcoholism and gastrointestinal hemorrhage or drugs (1,2). In developing countries, ALF was registered in 0.5% to 1% of all of patients with viral hepatitis B with mortality rate of 70%. Standard therapeutic strategies include treatment of infections and hemorrhage as well as supportive treatment of remote organ dysfunction such as hepatic encephalopathy (HE), renal failure, coagulation disorder, circulatory dysfunction and acute respiratory distress syndrome (ARDS) (3).

Randomized controlled trials have shown different long term survival rates in applying any of the commercially available extracorporeal liver support systems (4-6)

The most frequently used system is the Molecular Adsorbent Recirculating System (MARS) that dialyses patient's blood against a high-flux albumin filter. MARS is an extracorporeal liver support system based on albumin dialysis (7). By removing albumin-bound toxins that cause HE liver regeneration can be facilitated and multi-organ failure can be prevented (8,9). Here, we present a case of a successful application of MARS therapy in a critically ill patient suffering from ALF due to hepatitis B virus infection, not suitable for liver transplantation.

CASE REPORT

A 36-year-old Caucasian woman was admitted to ward of the Department of infectious disease with fever, reduced exercise tolerance, muscle and joint pain, right hypochondriac pain and vomiting for six days. Physical examination revealed a communicative, but confused patient, disoriented-in-time with body temperature of 38.3°C and a respiratory rate of 23 per minute. The yellow discoloration of the skin and sclera as well as signs of severe dehydration and hemodynamic instability were registered with arterial blood pressure (ABP) of 100/60 mmHg with Mean Arterial Pressure (MAP) of 80 mmHg and heart rate of 98 per minute. Moreover, peripheral edema and essential tremor of hands were confirmed. A Glasgow coma score (GCS) of 12 and Acute Physiology and Chronic Health Evaluation score II (APACHE II) of 10 were calculated.

Baseline biochemical parameters on admission are shown in table 1 (column 2).

Serological analysis was total anti-HBc antibodies, anti-HBe antibodies and anti-HBs antibodies positive. No evidence of HBsAg and HBeAg were registered (Table 2).

Based on these findings, ALF based on hepatitis B virus infection and stage II of HE were diagnosed and standard medical therapy (SMT) was initiated.

Parenteral rehydration with 30 mL per kilo of 0.9% of sodium chloride solution and 50 mL per hour of 10% of glucose solution was administered. The existing coagulation disorder was treated with 10 mL per kilo of fresh frozen plasma (total of 250 mL administered every 6 hours), 7 units of cryoprecipitate (according to the modified guideline for the administration of cryoprecipitate, 1 unit is administered on every 10 kilos in order to achieve a raise of fibrinogen concentration by 100 mg per deciliter) and 10 mg per day of Vitamin K. Nucleoside analog reverse transcriptase inhibitor, Lamivudine, was administered in dose of 100 mg per day. A 40 mg per 12 hours of proton pump inhibitor, pantoprazole, with 50 mL per hour of concentrated Nutrison® were applied.

Table 1. Baseline biochemical parameters on admission and after SMT

Parameters, units	Value on admission	Value after SMT	Normal range
1	2	3	4
pH, arterial blood	7.19	7.29	7.32-7.42
Lactate, mg/dL	135.1	135.1	1.8-19.8
Sodium, mEq/L	132	132	135-145
Activated clotting time (ACT), s	53	196	25.0-35.0
International Normalized Ratio (INR)	8.4	5.7	< 1.1
Fibrinogen, mg/dL	140	62.5	200-500
Alanine aminotransferase (ALT), IU/L	8 110	4 510	0-40
Aspartate aminotransferase (AST), IU/L	2 471	1 363	0-40
Total bilirubin, mg/dL	11.3	10.1	0.3-1.1
Direct bilirubin, mg/dL	6.02	5.4	0.1-0.3

Table 2. Serological examination reveals acute immune response that follows acute Hepatitis B viral infection

Serological test	Days of admission to Department			
	1	2	9	17
HBsAg	-	-	-	-
HBeAg	-	-	-	-
Anti-HBe anti-bodies	+	+	+	+
Anti-HBc anti-bodies IgM class	+	+	+	+
Anti-HBc anti-bodies IgG class	+	+	+	+
Anti-HBs anti-bodies	+	+	+	+

Biochemical parameters after SMT are shown in table 1 (column 3).

Despite the applied SMT, the patient was referred to the ICU 48 hours after admission due to HE progression and hemodynamic instability. The ABP of 60/40 mmHg with MAP of 50 mmHg required administration of 5 μg per kilo per minute of dopamine supportive stimulation was registered. Heart rate of 110 per minute was confirmed. After initial treatment with dopamine, MAP of 80 mmHg with stabilized heart function was confirmed. Furthermore, 0.5 grams per kilo of 20% of Mannitol solution were infused every 8 hours in order to prevent renal failure due to circulatory collapse. All the time in ICU, diuresis was normal (2000 to 3000 mL per day). Stage IV of HE was confirmed and a GCS of 3 and the Sequential Organ Failure Assessment score (SOFA score) of 12 were calculated. The Model for End-stage Liver Disease score (MELD-Na) of 37 points was calculated with estimated 3-months mortality rate of 52.6%.

In order to screen intracranial expansive masses as the possible causing factors of consciousness disorder a contrast-enhanced cranial CT scan was performed. Here, no brain edema or focal lesions were registered. CT scan was negative for intracranial pathological processes. Therefore, increased intracranial pressure was excluded.

Immediately after admission to the ICU, the patient was sedated, put on a respiratory tube and a central venous catheter was placed due to further medication's administration and intensive monitoring. Due to the rapid progression to multiorgan failure following ALF the patient was placed on extracorporeal liver dialysis using the MARS system approximately 6 hours after admission to the ICU.

MARS treatment was conducted in combination with a conventional hemodialysis machine (Prismaflex® System, Gambro Lundia AB, Lund, Sweden) with a standard buffered dialysis solution for continuous venovenous hemodiafiltration (CVVHDF). MARS device (MARS flux, Gambro Lundia AB, Lund, Sweden) consisted of an albumin-impregnated, highly permeable dialyzer with 600 mL of 20% serum albumin that was used to guarantee the removal of the toxins from the dialysate side. The albumin-enriched fluid was regenerated by perfusion through an anion exchanger column and an uncoated charcoal column and dialyzer for dialysis. The blood flow of the dialysis machine and the albumin dialysate circuit were both held at a median rate of 150 mL/min (interquartile range [IQR], 100–150 mL/min) on the albumin-impregnated membrane and ultrafiltration rate of 50 mL/min was established. The median dialysate flow rate was set to 2000 mL/min. Regional anticoagulation was performed by infusion of 4% trisodium citrate solution. The median citrate infusion rate, necessary to maintain the postfilter ionized calcium between 0.2 and 0.4 mmol/L, was 3.1 mmol/L (interquartile range, 2.3–4 mmol/L) blood flow. The median calcium chloride substitution rate was 0.9 mmol/L (0.3–1.7 mmol/L) dialysate. Total serum calcium remained stable during molecular adsorbent recirculating system treatments.

Since the value of the ACT of 196 seconds was registered, there was no need to administer any systemic anticoagulants.

The system was performed every day over 8 hours.

Using the MARS therapy in presented case we wanted to reduce serum levels of laboratory parameters that were correlated with ALF and accompanied with neurological improvement.

Table 3. Patient's biochemical parameters before and after MARS

Parameters, units	MARS Treatment cycle							
	I		II		III		IV	
	before	after	before	after	before	after	before	after
pH, arterial blood	7.29	7.34	7.58	7.58	7.54	7.46	7.42	7.40
Lactate, mg/dL	135.1	108.1	69.4	51.3	40.5	22.5	26.1	16.2
Sodium, mEq/L	132	139	140	140	136	139	141	141
Activated clotting time (ACT), s	196	74.2	122.5	64.3	58.1	106.7	41	37.1
International Normalized Ratio (INR)	5.71	5.71	3.36	2.91	1.93	1.63	1.41	1.36
Fibrinogen, mg/dL	62.5	58.2	58.8	77.9	73.8	93.2	99.3	104
Alanine aminotransferase (ALT), IU/L	4 510	3 960	2 371	1781	1 560	1 086	817	780
Aspartate aminotransferase (AST), IU/L	1 363	987	434	331	332	188	139	125
Total bilirubin, mg/dL	10.1	8.3	7.19	6.2	5.8	5.2	4.7	5.5
Direct bilirubin, mg/dL	5.4	4.2	4.0	3.85	3.56	3.02	1.88	1.26
BUN, mmol/L	16.5	13.9	7.7	7.2	6.8	4.3	4.8	4.2
Creatinine, μmol/L	110	97	63	43	57	42	57	53
SOFA score	12	13	11	10	10	9	10	9
GCS score	3	3	4	8	10	12	14	14
MELD score (points)	37	35	27	25	21	18	16	16
MELD score (estimated 3-month mortality, %)	52.6	52.6	19.6	19.6	19.6	6.0	6.0	6.0

Table 4. Plasma levels of α-fetoprotein

	Days after admission to the Department					
	2	4	6	8	10	14
α-fetoprotein, ng/mL	4.83	151.33	229.09	284.02	334.76	172.06

After four cycles of liver dialysis with MARS, treatment was disrupted because blood analysis revealed objective clinical improvement.

Biochemical parameters before and after every MARS treatment cycle are shown in table 3.

When MARS treatment was initiated, the patient was in stage IV of HE and with only registered reaction after very rough stimuli. After the second treatment cycle with MARS, stage III of HE was registered. Patient opened eyes on demand but without any signs of other communication skills. After the treatment procedure had been finished, patient was somnolent with established verbal communication. It was stage II of HE that was characterized with impaired awareness, especially regarding *place, time* or personal identity. MELD-Na score of 16 points was calculated with estimated 3-month mortality of 6.0%. After treatment with MARS, both SOFA and MELD-Na scores were significantly (for 25% and 83,8%, respectively).

Plasma levels of α-fetoprotein as a marker of liver regeneration are shown in table 4.

The patient has experienced what is referred to as an *extubation* after 9 days from ICU admission. After 14 days in ICU, the patient was referred to ward of the Department and no signs of catheter-related fever or sepsis, bleeding or mild thrombocytopenia as potential adverse events of MARS treatment were registered. On the 21st day after admission the patient was discharged from University Clinical center with serum levels of total bilirubin of 1.0 mg per deciliter and direct bilirubin of 0.17 mg per deciliter.

DISCUSSION

Acute liver failure (ALF), the original term "fulminant hepatic failure, (FHF)", is defined as a severe liver injury, potentially reversible in nature and with onset of hepatic encephalopathy (HE) within eight weeks of the first symptoms in the absence of pre-existing liver disease (1,10). Although it can occur from various causes, ALF results from massive necrosis of hepatocytes with appearance of progressive jaundice, HE and coagulation disorder of different degree. ALF is divided into hyper acute, acute and sub-acute forms according to the time between the onset of symptoms and the HE development. In hyper acute cases, this interval is a week or less and is commonly caused by viral infection (1,11). This interval provides clues to the cause of disease, but more likely complications and prognosis with supportive medical care (1,12). In reported case, hyper acute form of the disease was registered within the six days after initial symptoms were developed. This period is associated with a better survival prognosis.

Only 8% of almost all cases of ALF were originated from acute hepatitis B viral infections (13). This infection was a reasonable cause of ALF in our presented case. Very strong immune response on infection can also promote ALF and that was described in presented case.

After initial SMT had been administered, HE progressed rapidly in this case. We administered an endotracheal intubation and sedation for airway control in order to facilitate general care and control of oxygen. These procedures made liver transplantation impossible as surgery became contraindicated because of rapidly progression to multiorgan failure and dramatic deterioration of patient's medical condition. Therefore, the patient could not be a proper candidate for a liver transplantation and this intervention was not considered as a treatment option.

Kidney dysfunction with elevated plasma level of blood urea nitrogen (BUN) and creatinine did not indicate a hepatorenal syndrome (HRS) but the state of circulatory collapse and subsequent hemoconcentration. Therefore, Common Renal Replacement Therapy (RRT) was not considered as an appropriately treatment procedure in this case. Furthermore, RRT would only remove in-water soluble toxins but not albumin-conjugated. Recent studies also suggested that in patient with ALF and subsequent kidney dysfunction who are not candidates for liver transplantation (LT), RRT would not be beneficial (14,15). Moreover, RRT has not been shown to significantly alter outcomes in patients with ALF and kidney dysfunction in the absence of LT (16).

Although Molecular Adsorbent Recirculating System (MARS) is not recommended a standardized treatment option for ALF caused by acute Hepatitis B viral infections, MARS was introduced first time in our facility. It was done in order to stabilize the patient and to allow liver to recover from failure or to "bridge" patient to safer LT. Significant changes in plasma levels of several biochemical parameters were seen already after initial MARS administration compared to baseline (lactate, coagulation parameters, ALT, AST and bilirubin).

Low plasma level of α-fetoprotein (AFP) is one of the risk factor that promote poor survival prognosis in ALF (17). An increase in α-fetoprotein (AFP) following ALF is considered indicative of hepatic regeneration. Patients with ALF usually present with increased serum levels of AFP during hospitalization (18-20). AFP is thought to be secreted from liver progenitor cells (LPCs) as LPCs, but not mature hepatocytes (MHs) express AFP (20,21). Furthermore, an increase of serum level of AFP after admission in ICU is associated with the better prognosis of patients with ALF (18,20). Therefore, liver regeneration

is considered to play an important role in the recovery of the liver function and increasing the liver volume. In addition, AFP seems to be potent marker of better prognosis after ALF.

The safety profile of MARS is remarkable for an extracorporeal circuit. Transient no significant hyperbilirubinemia had been observed in presented case after the fourth treatment cycle, which was probably not due to MARS although the serum total and direct bilirubin levels on the discharge were normal. Bañares et al. also suggested that most studies had not reported any significant adverse effects, except for mild thrombocytopenia, which did not have any clinical implications (6). Sponholz et al. also demonstrated that the investigated albumin dialysis procedures were safe for temporary extracorporeal liver support (22).

CONCLUSION

We suggest that the MARS treatment can be the treatment option for patients in ICU with ALF associated with HE grade 3 or 4, especially in terms of the low likelihood of liver transplantation. It is significant method of treatment in developing countries where liver transplantation is impossible due to the lack of liver transplants. We also show that albumin dialysis with the MARS system is a safe procedure that provides support of organ failure (liver, kidney, and brain) in ALF. The MARS method allows the liver to regenerate completely after ALF. Despite the lack of large clinical trials on the use of MARS in treatment of ALF caused by viral infections, the literature reviews suggest that there are clinical and biological benefits from this treatment method. Furthermore, it can bypass liver failure patients to LT and reduce overall treatment expenditures.

Conflict of interest

The authors declare that they have no conflict of interest and financial support

REFERENCES

1. Bernal W, Wendon J. Acute Liver Failure. N Engl J Med 2013; 369: 2525-34.
2. Blasco-Algora S, Masegosa-Ataz J, Gutiérrez-García ML, López SA, Fernández-Rodríguez CM. Acute-on-chronic liver failure: Pathogenesis, prognostic factors and management. World J Gastroenterol 2015; 21(42): 12125-40.
3. Olin P, Hausken J, Foss A, Karlsen TH, Melum E et Haugaa H. Continuous molecular adsorbent recirculating system treatment in 69 patients listed for liver transplantation. Scandinavian Journal of Gastroenterology. 2015; 50: 1127-34.
4. Krisper P, Stadlbauer V, Stauber RE. Clearing of toxic substances: are there differences between the available liver support devices? Liver Int 2011; 31: 5–8.
5. Faybik P, Krenn CG. Extracorporeal liver support. Curr Opin Crit Care 2013; 19: 149–53.
6. Banares R, Nevens F, Larsen FS, Jalan R, Albillos A, Dollinger M, et al. Extracorporeal albumin dialysis with the molecular adsorbence recirculating system in acute-onchronic liver failure: the RELIEF trial. Hepatology 2013; 57: 1153–62.
7. Cisneros-Garza LE, del Rosario Muñoz-Ramírez M, Muñoz-Espinoza LE, Ruiz Velasco JAV, Moreno-Alcántar R, Marín-López E. The molecular adsorbent recirculating system as a liver support system. Summary of Mexican experience. Ann Hepatol 2014; 13(2): 240-7.
8. Pares A, Deulofeu R, Cisneros L, Escorsell A, Salmeron JM, Caballeria J, et al. Albumin dialysis improves hepatic encephalopathy and decreases circulating phenolic aromatic amino acids in patients with alcoholic hepatitis and severe liver failure. Crit Care 2009; 13: R8.
9. Chiu A, Tsoi NS, Fan ST. Use of the molecular adsorbents recirculating system as a treatment for acute decompensated Wilson disease. Liver Transpl 2008; 14: 1512–16.
10. Trey C, Davidson CS. The management of fulminant hepatic failure. Prog Liver Dis 1970; 3: 282-98.
11. Wlodzimirow KA, Eslami S, Abu- Hanna A, Nieuwoudt M, Chamuleau RA. Systematic review: acute liver failure — one disease, more than 40 definitions. Aliment Pharmacol Ther 2012; 35: 1245-56.
12. Mochida S, Nakayama N, Matsui A, Nagoshi S, Fujiwara K. Re-evaluation of the Guideline published by the Acute Liver Failure Study Group of Japan in 1996 to determine the indications of liver transplantation in patients with fulminant hepatitis. Hepatol Res 2008; 38: 970-9.
13. Wai CT, Fontana RJ, Polson J, Hussain M, Shakil AO, Han SH, et al. Clinical outcome and virological characteristics of hepatitis B-related acute liver failure in the United States. J Viral Hepat 2005; 12(2): 192–8.
14. Brochard L, Abroug F, Brenner M et al. An Official ATS/ERS/ESICM/SCCM/SRLF Statement:Prevention and management of acute renal failure in the ICU patient: An international consensus conference in intensive care medicine. Am J Resp Crit Care Med 2010; 181: 1128-55.
15. Kelly JA, Lameire N and the KDIGO Work Group. KDIGO Clinical Practice Guideline for Acute kidney injury. Kidney International 2012; 2(1).
16. Wadei H, Mai M, Ahsan N, Gonwa T. Hepatorenal syndrome: patho- physiology andmanagement. Clin J Am Soc Nephrol 2006; 1: 1066-79.
17. Ferenci P, Lockwood A, Mullen K, et al. Hepatic encephalopathy: definition, nomenclature, diagnosis, and quantification. Hepatology 2002; 35: 716-21.

18. Schiodt FV, Ostapowicz G, Murray N et al. Alpha-fetoprotein and prognosis in acute liver failure. Liver Transplant 2006; 12: 1776-81.
19. Schmidt LE, Dalhoff K. Alpha-fetoprotein is a predictor of outcome in acetaminophen-induced liver injury. Hepatology 2005; 41: 26–31.
20. Kakisaka K, Kataoka K, Onodera M, Suzuki A, Endo K, Tatemichi Y et al. Alpha-fetoprotein: A biomarker for the recruitment of progenitor cells in the liver in patients with acute liver injury or failure, Hepatology Research 2015; 45(10): E12-E20.
21. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. Hepatology 2006; 43: S45–53.
22. Sponholz C, Matthes K, Rupp D, Backaus W, Klammt S, Karailieva D et al. Molecular adsorbent recirculating system and single-pass albumin dialysis in liver failure – a prospective, randomised crossover study. Critical Care 2016; 20(1): 2

OBSTRUCTIVE HYPERTROPHIC CARDIOMYOPATHY WITH CONCOMITANT MITRAL REGURGITATION TREATED WITH A SEPTAL MYECTOMY AND MV REPAIR: A CASE REPORT

Irena Mitevska[1], Elizabeta Srbinovska[1], Marijan Bosevski[1], Sasko Jovev[2], Omer Dzemali[3]
[1] University Cardiology Clinic, Skopje, Macedonia
[2] Cardiac Surgery Clinic, Skopje, Macedonia
[3] Stadtspital Triemli Zürich, Switzerland

OPSTRUKTIVNA HIPERTROFIČNA KARDIOMIOPATIJA SA PRIDRUŽENOM MITRALNOM REGURGITACIJOM KOJA JE LEČENA SEPTALNOM MIJEKTOMIJOM I REPARACIJOM MITRALNOG ZALISTKA: PRIKAZ SLUČAJA

Irena Mitevska[1], Elizabeta Srbinovska[1], Marijan Bosevski[1], Sasko Jovev[2], Omer Dzemali[3]
[1] Univerzitetska kardiološka klinika, Skopje, Makedonija
[2] Klinika za kardiohirurgiju, Skopje, Makedonija
[3] Bolnica „Triemli" Cirih, Švajcarska

Received / Primljen: 11. 08. 2017. Accepted / Prihvaćen: 28. 08. 2017.

ABSTRACT

Case presentation

Hypertrophic cardiomyopathy (HCM) is the most common and very heterogeneous genetic cardiac disease with a different clinical presentation and prognosis. The overall prevalence of the disease is estimated between 0.05-0.2% of the population. Left ventricular outflow obstruction at rest is present in about 20% of patients. Most of the patients have a normal life expectancy, however high risk patients might develop heart failure, atrial fibrillation, ventricular arrhythmias and sudden cardiac death.

We present the case of 47-year-old Caucasian man who was hospitalized at our clinic with a history of chest pain and shortness of breath on physical activity in the last six months, which caused significant limitations of his life quality. Hypertrophic obstructive cardiomyopathy was diagnosed in 2011, when the patient was put on therapy with beta blocker. Transthoracic echocardiography revealed normal systolic function, presence of systolic anterior mitral valve motion (SAM) with moderate mitral regurgitation (MR). There was a significant concentric left ventricular hypertrophy predominantly located in the ventricular septum. The intraventricular gradient at rest was 77.8 mmHg. MRI of the heart confirmed significant LV hypertrophy with regions of fibrosis at the septum. The patient shortness of breath worsened progressively in the last month (NYHA III) despite optimized medical treatment with maximal beta blocker dose. Surgical approach with septal myectomy was performed with mitral valve repair. There were no operative complications, with excellent postoperative recovery and complete symptoms resolution. Control Doppler echocardiograms revealed LVOT rest gradient reduction to 34 mmHg. The good operative results were still present 9 months after the intervention.

Our case confirmed that septal myectomy with MV repair is an excellent treatment approach in young patient with obstructive hypertrophic cardiomyopathy and mitral valve involvement refractory to medical treatment.

Keywords: *Hypertrophic cardiomyopathy, septal myectomy, mitral valve repair*

SAŽETAK

Hipertrofična kardiomiopatija (HCM) je najčešće i vrlo heterogeno genetsko oboljenje srca sa kliničkom slikom koja može znatno da varira i različitom prognozom. Ukupna prevalenca ovog oboljenja se procenjuje na 0,05-0,2% populacije. Opstrukcija izbacivanja krvi iz leve komore postoji u oko 20% pacijenata. Kod većine pacijenata se očekuje uobičajeno trajanje života, ali kod pacijenata sa visokim rizikom može da se razvije srčana slabost, fibrilacija pretkomora, aritmije komora i iznenadna srčana smrt.

Prikazan je slučaj belca starosti 47 godina koji je primljen u našu bolnicu zbog bola u grudima i osećaja gubitka daha tokom fizičke aktivnosti u poslednih 6 meseci, što je uzrokovalo znatno smanjenje kvaliteta života pacijenta. Hipertrofična, opstruktivna kardiomiopatija je dijagnostikovana 2011. godine, kada su pacijentu u terapiju uvedeni beta blokatori. Transtorakalnom ehokardiografijom je utvrđena fiziološka sistolna funkcija, postojanje antriornog kretanja mitralne valvule u sistoli sa umerenom mitralnom regurgitacijom. Postojala je znatna koncentrična hipertrofija leve komore pretežno u oblasti međukomorskog septuma. Intavenrikularni gradijent u mirovanju je iznosio 77,8 mmHg. Pregledom magnetnom rezonacom potvrđena hipertrofija leve komore sa poljima fibroze u septumu. Osećaj gubitka daha kod pacijenta se značajno pogoršao (NYHA III) u poslednjih mesec dana uprkos primeni maksimalne doze beta blokatora. Izvršena je hirurška mijektomija septuma uz reparaciju mitralnog zalistka. Nije bilo komplikacija tokom operacije, postoperativni oporavak je bio izvrstan uz potpuni gubitak simptoma. Na kontolnim Doppler ehokardiogramima povrđeno je smanjenje gradijenta u izlaznom traktu leve komore na 34 mmHg. Dobri postoperativni rezultati su postojali i 9 meseci nakon operacije.

Ovaj slučaj potvrđuje da je mijektomija septuma uz reparaciju mitralnog zalistka odličan terapijski pristup u lečenju mlađih pacijenata sa opstruktivnom hipertrofičnom kardiomiopatijom i insuficijencijom mitralnog zalistka kada postoji rafraktarnost na medikamentozno lečenje.

Ključne reči: *Hipertrofična kardiomiopatija, septalna mijektomija, reparacija mitralnog zalistka.*

sciendo

UDK: 616.127-089 / Ser J Exp Clin Res 2018; 19 (2): 195-200
DOI: 10.2478/SJECR-2018-0017

Corresponding author: Irena Mitevska
University Cardiology Clinic; Ul.Vodnjanska 17, 1000 Skopje, Macedonia
Mob: +389 75 491 21; E-mail: peovskai@yahoo.com

INTRODUCTION

Hypertrophic cardiomyopathy (HCM) is a very heterogeneous genetic cardiac disease with a different clinical presentation and prognosis. The overall prevalence of the disease is estimated between 0.05-0.2% of the population (1). The disease is characterized by asymmetric hypertrophy of the left ventricular (LV) and variable degrees of dynamic left ventricular outflow tract obstruction (LVOTO) due to the systolic anterior motion (SAM) of the anterior mitral leaflet. LV hypertrophy can be localized in the basal, mid or apical part of the septum. Left ventricular outflow obstruction at rest is present in about 20% of patients. Most patients achieve a normal life expectancy. On the other hand, in some patients, HCM is associated with disease complications which can result in disease deterioration or premature death.

There are three pathways of clinical progression: sudden cardiac death due to ventricular tachyarrhythmia, heart failure and atrial fibrillation associated with an increased risk of systemic thromboembolism and stroke. Treatment strategy is based on medical therapy, especially with the use of β blockers and calcium-channel blockers , life style and physical activity modification (1,2). Septal myectomy is the first treatment of choice in young patients who have LVOT obstruction at rest >50mmHg and are symptomatic despite maximal medical therapy, with excellent results in experienced centers and properly selected patients (3).

In this report, we present a case of young symptomatic patient with marked hypertrophic obstructive cardiomyopathy with mitral valve regurgitation treated with septal myectomy and mitral valve repair.

CASE REPORT

A 47-year-old Caucasian man was hospitalized at University Cardiology Clinic in Skopje Clinical Center in October 2016 due to chest pain and severe dispnea. Patient reported the history of chest pain (Canadian Cardiovascular Society (CCS) class II) in the last year and shortness of breath on physical activity, which intensified in the last few days before the admission, which caused significant limitations of his life quality.

Hypertrophic obstructive cardiomyopathy was diagnosed in 2011, when the patient was put on therapy with beta blocker Bisoprolol 5mg daily. He had no history of coronary artery disease (CAD), and no risk factors for CAD, and no history of syncope or palpitations. There was no sudden cardiac death in the family members. Patient risk score for 5 year sudden cardiac death was 2.6, and there was no indication for preventive implantation of ICD.

His physical examination showed systolic murmur over aortic valve and lateral sternum. ECG showed left bundle branch block, with heart rate 65 bpm. His blood pressure was 120/70mmHg. All laboratory findings, lipids values and hs-Troponin T were within normal limits. Pulmonary functions (pulmonary X-ray, spirometry and gas analyses) were normal.

Transthoracic echocardiography revealed normal systolic function with EF 76%, left ventricular (LV) end diastolic dimension 53mm, LV end systolic dimension 28 mm. There was a presence of systolic anterior mitral valve motion (SAM) with moderate mitral regurgitation (MR) due to SAM and mild posterior valve prolaps (Carpentier II) with moderate left atrial enlargement (LA 43mm, LA volume 58ml). He had diastolic dysfunction of pseudonormalization type (DT 216ms; E/A 1,1; E' 0,06m/s; E/E' 15,9) and severe reduction of global longitudinal LV function (LV global longitudinal strain was -12%). The right ventricle had normal size and function with TAPSE 22, FAC 38%. There was significant concentric left ventricular hypertrophy with predominant location in the ventricular septum with septal thickness of 32mm. Dimension of the posterior wall was 17mm. The intraventricular gradient at rest was 77.8mmHg, provocable gradient on Valsalva maneuver was 95mmHg. MRI of the heart confirmed significant LV hypertrophy with regions of fibrosis at the septum. Coronary angiography revealed normal coronary arteries with venticulography showing significant asymmetric hypertrophy, located predominately at the septum.

The patient shortness of breath worsened progressively in the last month (NYHA III) despite receiving medical treatment with maximal tolerated beta blocker dose (Bisoprolol 7,5mg total daily dose). The case was discussed together with the cardiac surgery team and the indication for septal myectomy was established.

The operation was performed in December 2016. The procedure was fully described to the patient, and a written informed consent was obtained before the operation. Surgical transaortic approach under cardiopulmonary bypass was first performed. Preoperative transesophageal echocardiography (TEE) was performed for detailed evaluation of LVOT obstruction and mitral valve apparatus. Conventional septal myectomy was undertaken to relieve SAM and LVOT obstruction. Mitral valve repair - edge to edge procedure sec. Alfierie and mitral ring annuloplasty with implantation of mitral ring type CG future- Medtronic size 32 followed. The procedure was performed at our clinic by visiting professor, cardiac surgeon from Triemli City Hospital in Zurich. There were no operative complications, with excellent postoperative recovery and complete symptoms resolution.

Patient was discharged in good condition and no symptoms after 8 days. Control Doppler echocardiogram before the discharge revealed LVOT rest gradient reduction to 34mmHg. The good operative results were still present 9 months after the intervention with no gradient increase. Patient therapy is beta blocker Bisoprolol 5mg daily. He is asymptomatic with normal physical activity assessed on the last clinical visit in august 2017. ECG and echocardiography screening was performed on the close family members and we have not found any signs of HCM presence.

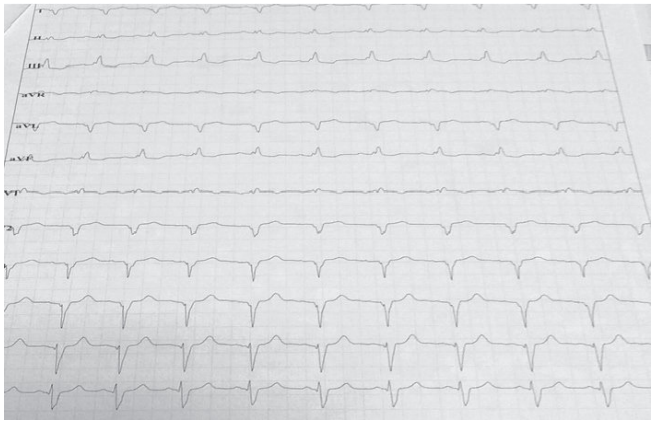


Figure 1.
Rest ECG showing left bundle branch block with heart rate 65bpm

DISCUSSION

Septal myectomy has been established as a gold standard treatment for the relief of left ventricular outflow tract obstruction and cardiac symptoms in patients with obstructive hypertrophic cardiomyopathy with drug refractory functional limitations (4). The pharmacologic treatment of HCM is based on β blockers, calcium-channel blockers and disopyramide. Although these medications improve symptoms, there is no evidence they influence prognosis in patients with LVOT obstruction. The biggest revolution in the HCM management is septal reduction therapy, despite the fact that this approach is used is the selected and relatively small number of symptomatic patients with LVOT obstruction at rest.

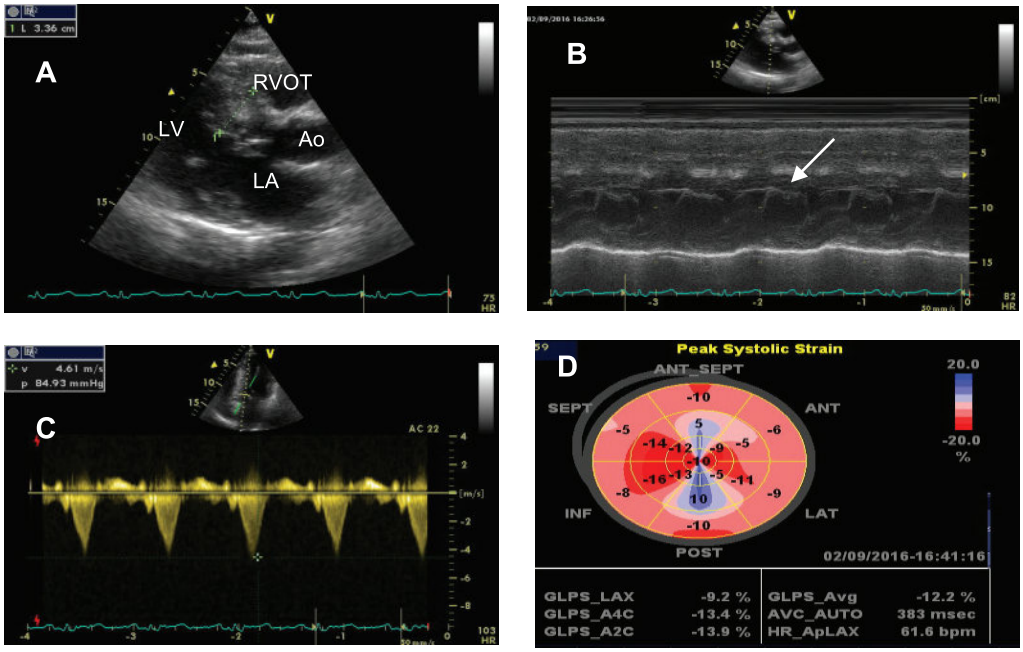


Figure 2. Two-dimensional echocardiograms. **(A)** Longitudinal, long axis view of the LV shows left ventricular hypertrophy confined to ventricular septum with regions of fibrosis. **(B)** M-mode of mitral valve showing the presence of systolic anterior mitral valve motion (SAM)- white arrow. **(C)** Presence of severe rest intraventricular gradient 84.93mmHg. **(D)** Severely reduced global longitudinal left ventricular strain (-12%) Ao, Aorta; LA, Left atrium; LV Left ventricle; RVOT, Right ventricle outflow tract.

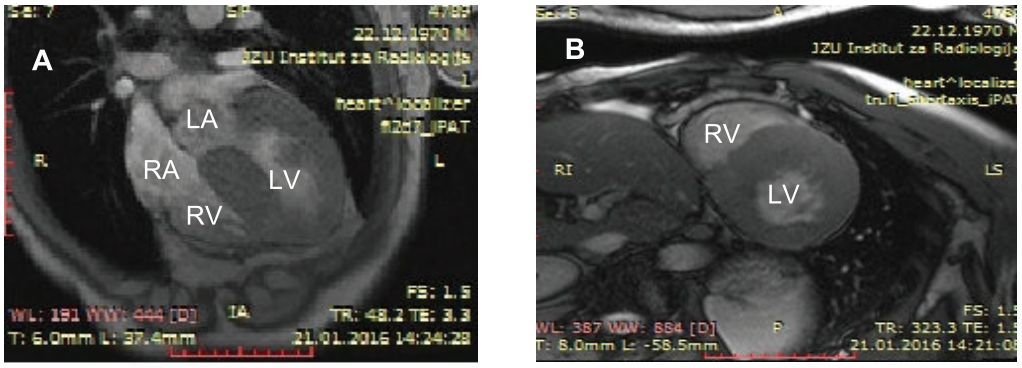


Figure 3
Nuclear magnetic resonance images of the patient. **(A)** Long-axis, four-chamber nuclear magnetic resonance (NMR) scan taken in end-systole shows hypertrophy confined to the left ventricular (LV) septum. **(B)** Short axis chamber view nuclear magnetic resonance (NMR) shows marked septal and lateral walls hypertrophy. LA, Left atrium; RA, Right atrium; RV, Right ventricle.

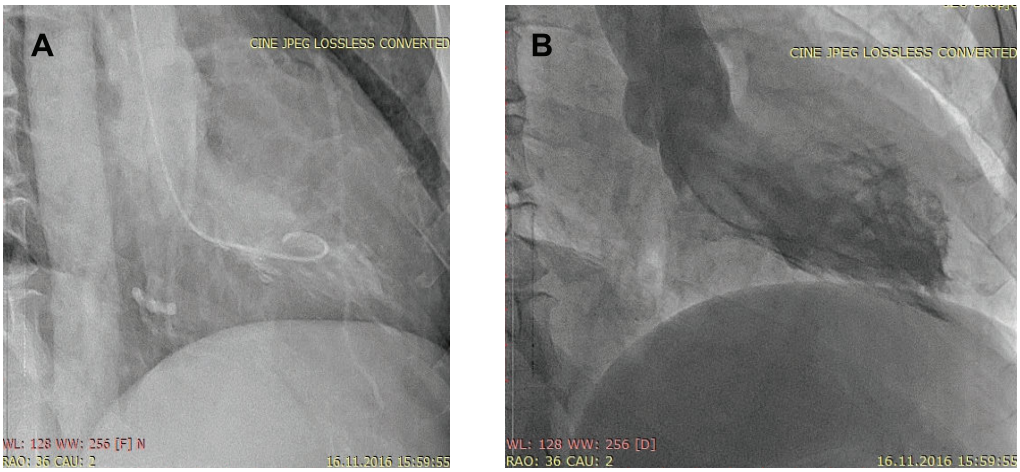


Figure 4
Cardiac catheterization ventriculograms. (A) Ventriculogram of the left ventricle shows severe left ventricular hypertrophy. **(B)** Ventriculogram of the left ventricle shows banana shaped left ventricle

Still, one of the most controversial issues in the treatment approach in patients with HCM concerns the use of percutaneous alcohol septal ablation (ASA) which causes a region of myocardial infarction aimed to reduce outflow tract obstruction (5). This procedure is dominating septal reduction treatment option in Europe. The scientific data suggests that although this procedure is associated with high success rate, high incidence of heart block and pacemaker implantation, arrhythmia risk, as well as less predictive and smaller final gradient reduction, position ASA as procedure with higher overall procedural complication rates compared to myectomy (6). On the basis of the latest scientific data, about 10-12% of the patients after ASA experience significant ventricular arrhythmias. Since myectomy does not produce intramyocardial scarring and fibrosis, there is no evidence that this procedure increases the risk of arrhythmias and left ventricular dysfunction. This method has advantages over septal alcohol ablation in younger patients with severe septal hypertrophy. One of the obvious advantages of myectomy is direct visualization of complex LV outflow tract anatomy with all structural abnormalities that lead to mechanical subaortic obstruction, mitral apparatus function and morphology. The concern that the mitral valve leaflets abnormalities can produce dynamic outflow obstruction, even after septal muscular resection, has led several surgeons to supplement myectomy with mitral valve repair in selected individuals (7-9). The aim of septal myectomy is to relieve SAM of the mitral valve as a cause of obstruction, not only to enlarge the LVOT anatomically. Frequently in some patients septal myectomy is modified with extended myectomy, partial excision of papillary muscles, mitral valve and subvalvular interventions when isolated septal myectomy is thought to be fully adequate. The choice of optimal procedure must be individualized based on age, comorbidities, degree of hypertrophy, suitability of coronary anatomy, primary mitral valve disease, patient preferences and operator experience. The standard septal myectomy (Marrow procedure) is not always an optimal procedure in every patient, especially with the mitral valve involvment. Structural and functional mitral valve and subvalvular abnormalities have been reported in patients with LVOT obstruction and HCM. The most frequent abnormalities are papillary muscle insertion directly into the anterior mitral leaflet, abnormal chordae tendineae attached to the ventricular septum or free wall and accessory papillary muscles, which may push the mitral leaflets toward the septum and produce LVOT obstruction. Most of the patients with concomitant abnormalities of the mitral valve and subvalvular apparatus can be managed without mitral valve replacement, and also other cardiac lesions can be repaired simultaneously. Additional operative procedures includes anterior mitral leaflet excision, “false chordae” excision and papillary muscle realignment.The most recent data from experienced centers are reporting low operative mortality for isolated septal myectomy (approximately 1%). Septal myectomy also showed excellent late procedure results with over 90% of patients improving in NYHA class, which persisted in the follow-up period. Late survival in patients with obstructive hypertrophic cardiomyopathy treated with myectomy is similar to the same age healthy individuals (10-12). Additionally, it is reported the myectomy may be associated with decreased long-term risk of sudden cardiac death.

European and American guidelines on the management of HCM recommend that myectomy should be the preferred intervention for obstructive HCM in patients below 50 years (1). Over the past three decades, the technique for septal myectomy has evolved from the classic Morrow myectomy, to a more extensive left ventricular septal myectomy. Interventions are guided by intraoperative transesophageal echocardiography (TEE) with special attention to the septal anatomy and thickness, and mitral valve function (13). Data from the scientific literature shows long-term survival in patients with HCM after septal myectomy, is 99%, 98% and 95%, at 1, 5 and 10 years, accordingly. However, surgical myectomy does not reduce the need to assess

each patient's risk for sudden cardiac death and to consider appropriate treatment modalities based on the SCD risk in individual patients (13). Mayo Clinic experience long term follow-up data shows that majority of patients will have an excellent symptomatic benefit and be able to have normal quality of life (14, 15).

HCM is a disease characterized by unexplained, marked and asymmetric left ventricular (LV) hypertrophy associated with non dilated ventricular chambers in the absence of another cardiac or systemic disease that are able to produce the magnitude of hypertrophy that fulfills the criteria for HCM (1). Clinically, HCM is usually recognized by a maximal LV wall thickness >15 mm. However, it should be underscored that in principle, any degree of wall thickness is compatible with the presence of the HCM genetic substrate. Echocardiography is the established method of choice for the diagnosis and prognosis of HCM, but also helps resolve several diagnostic challenges in HCM. The method enables differentiation of HCM from athlete's heart, left ventricular hypertrophy in hypertension and aortic stenosis, isolated basal septal hypertrophy in adults, apical forms of HCM, non-compaction cardiomyopathy and infiltrative myocardial disease. In order to establish the diagnosis of HCM, a systematic echocardiography approach is necessary. The echocardiography examination should include confirming of LV hypertrophy, assessment of LVOT obstruction, systolic anterior motion - SAM, mitral valve apparatus assessment, assessment of systolic and diastolic LV function and left atrial size. Strain imaging assesses subclinical LV dysfunction even in the presence of normal LVEF (15). The following two-dimensional (2D) echocardiography criteria are used to aid diagnosis: 1. Unexplained maximal wall thickness >15 mm in any myocardial segment, or 2. Septal/posterior wall thickness ratio >1.3 in normotensive patients, or 3. Septal/posterior wall thickness ratio >1.5 in hypertensive patients (16).

It is clinically important to distinguish between the obstructive and nonobstructive forms of HCM because management strategies depend on the presence or absence of symptoms caused by obstruction. For HCM, it is the peak instantaneous LV outflow gradient rather than the mean gradient that influences treatment decisions. Up to one third of patients with HCM have rest LVOT obstruction (defined as gradients ≥30 mm Hg). Another one third have physiologically provoked gradients (<30 mm Hg at rest and ≥30 mm Hg with physiologic provocation) (16,17). The final one third have the nonobstructive form of HCM (gradients <30 mm Hg at rest and with provocation). Marked gradients ≥50 mm Hg, either at rest or with provocation, represent the conventional threshold for surgical or percutaneous intervention if symptoms cannot be controlled with medications (16,17).

Magnetic resonance imaging is also important imaging modality in HCM. The extensity of myocardial fibrosis has prognostic implications.

We have presented a case of young symptomatic patient with obstructive HCM and moderate MR, who was successfully treated with septal myectomy and mitral valve repair. LVOT gradient fall to 25mmHg. He has fast clinical improvement, LVOT gradient reduction and excellent quality of life during the 9 months follow up.

CONCLUSION

Our case confirmed that surgical myectomy in combination with mitral valve repair in obstructive HCM and moderate MR is safe and improves quality of life performed by experienced surgeon. In accordance with latest guidelines and consensus panel recommendations, the presented case support septal myectomy as the excellent treatment choice for HCM in young patients with severe drug-refractory symptoms caused by LV outflow obstruction.

Conflict of interests:
Not declared

REFERENCES

1. 2014 ESC Guidelines on diagnosis and management of hypertrophic cardiomyopathy: the Task Force for the Diagnosis and Management of Hypertrophic Cardiomyopathy of the European Society of Cardiology (ESC). Eur Heart J 2014; 35(39):2733-79.
2. Prevalence of hypertrophic cardiomyopathy in a general population of young adults. Echocardiographic analysis of 4111 subjects in the CARDIA Study. Coronary Artery Risk Development in (Young) Adults Maron BJ, Gardin JM, Flack JM, et al.. Circulation. Aug 15 1995;92(4):785-9.
3. Hypertrophic cardiomyopathy: current understanding and treatment objectives. Soor GS, Luk A, Ahn E, Abraham JR, Woo A, Ralph-Edwards A, et al. J Clin Pathol. Mar 2009;62(3):226-35.
4. Maron BJ. Controversies in cardiovascular medicine. Surgical myectomy remains the primary treatment option for severely symptomatic patients with obstructive hypertrophic cardiomyopathy. Circulation 2007;116:196–206.
5. Leonardi RA, Kransdorf EP, Simel DL, Wang A. Meta-analyses of septal reduction therapies for obstructive hypertrophic cardiomyopathy: comparative rates of overall mortality and sudden cardiac death after treatment. Circ Cardiovasc Interv 2010;3:97–104.
6. Merrill WH, Friesinger GC, Graham TP Jr, Byrd BF 3rd, Drinkwater DC Jr, Christian KG, Bender HW Jr. Long-lasting improvement after septal myectomy for hypertrophic obstructive cardiomyopathy. Ann Thorac Surg 2000;69: 1732–1735.
7. McIntosh CL, Maron BJ, Cannon RO, Klues HG. Initial results of combined anterior mitral leaflet plication and ventricular septal myotomy-myectomy for relief of left ventricular outflow tract obstruction in patients with hypertrophic cardiomyopathy. Circulation 1992;86:II60–II67.

8. Matsui Y, Shiiya N, Murashita T, Sasaki S, Yasuda K. Mitral valve repair and septal myectomy for hypertrophic obstructive cardiomyopathy. J Cardiovasc Surg 2000; 41:53–56.
9. Kofflard MJ, van Herwerden LA,Waldstein DJ, Ruygrok P, Boersma E, Taams MA, ten Cate FJ. Initial results of combined anterior mitral leaflet extension and myectomy in patients with obstructive hypertrophic cardiomyopathy. J Am Coll Cardiol 1996;28:197–202.
10. Maron BJ, McKenna WJ, Danielson GK, Kappenberger LJ, Kuhn HJ, Seidman CE, Shah PM, Spencer WH, Spirito P, ten Cate FJ, Wigle ED. American College of Cardiology/European Society of Cardiology Clinical Expert Consensus Document on Hypertrophic Cardiomyopathy: A report of the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents and the European Society of Cardiology Committee for Practice Guidelines. J Am Coll Cardiol. 2003 Nov 5;42(9):1687-713.
11. Echocardiography in the treatment of hypertrophic cardiomyopathy Musat D, Sherrid MV. Anadolu Kardiyol Derg. Dec 2006;6 Suppl 2:18-26.
12. Woo A, Williams WG, Choi R, Wigle ED, Rozenblyum E, Fedwick K, Siu S,Ralph-Edwards A, Rakowski H. Clinical and echocardiographic determinants of long-term survival after surgical myectomy in obstructive hypertrophic cardiomyopathy. Circulation 2005;111:2033–2041.
13. Ommen SR, Maron BJ, Olivotto I, Maron MS, Cecchi F, Betocchi S, Gersh BJ, Ackerman MJ, McCully RB, Dearani JA, Schaff HV, Danielson GK, Tajik AJ, Nishimura RA. Long-term effects of surgical septal myectomy on survival in patients with obstructive hypertrophic cardiomyopathy. J Am Coll Cardiol 2005; 46:470–476.
14. Dearani JA, Ommen SR, Gersh BJ, Schaff HV, Danielson GK. Surgery insight: septal myectomy for obstructive hypertrophic cardiomyopathy—the Mayo Clinic experience. Nat Clin Pract Cardiovasc Med 2007;4:503–512.
15. Leonardi RA, Kransdorf EP, Simel DL, Wang A. Meta-analyses of septal reduction therapies for obstructive hypertrophic cardiomyopathy: comparative rates of overall mortality and sudden cardiac death after treatment. Circ Cardiovasc Interv 2010;3:97–104.
16. 2011 ACCF/AHA Guidelines for the Diagnosis and Treatment of Hypertrophic Cardiomyopathy: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Developed in collaboration with the American Association for Thoracic Surgery, American Society of Echocardiography, American Society of Nuclear Cardiology, Heart Failure Society of America, Heart Rhythm Society, Society for Cardiovascular Angiography and Interventions, and Society of Thoracic Surgeons. Gersh BJ, Maron BJ, Bonow RO, et al. J Thorac Cardiovasc Surg. 2011 Dec;142(6):e153-203.
17. Echocardiography in hypertrophic cardiomyopathy: the role of conventional and emerging technologies. Afonso LC, Bernal J, Bax JJ, Abraham TP. JACC Cardiovasc Imaging. 2008 Nov;1(6):787-800.

INSTRUCTION TO AUTHORS FOR MANUSCRIPT PREPARATION

Serbian Journal of Experimental and Clinical Research is a peer-reviewed, general biomedical journal. It publishes original basic and clinical research, clinical practice articles, critical reviews, case reports, evaluations of scientific methods, works dealing with ethical and social aspects of biomedicine as well as letters to the editor, reports of association activities, book reviews, news in biomedicine, and any other article and information concerned with practice and research in biomedicine, written in the English.

Original manuscripts will be accepted with the understanding that they are solely contributed to the Journal. The papers will be not accepted if they contain the material that has already been published or has been submitted or accepted for publication elsewhere, except of preliminary reports, such as an abstract, poster or press report presented at a professional or scientific meetings and not exceeding 400 words. Any previous publication in such form must be disclosed in a footnote. In rare exceptions a secondary publication will acceptable, but authors are required to contact Editor-in-chief before submission of such manuscript. the Journal is devoted to the Guidelines on Good Publication Practice as established by Committee on Publication Ethics-COPE (posted at www.publicationethics.org.uk).

Manuscripts are prepared in accordance with „Uniform Requirements for Manuscripts submitted to Biomedical Journals“ developed by the International Committee of Medical Journal Editors. Consult a current version of the instructions, which has been published in several journals (for example: Ann Intern Med 1997;126:36-47) and posted at www.icmje.org, and a recent issue of the Journal in preparing your manuscript. For articles of randomized controlled trials authors should refer to the „Consort statement“ (www.consort-statement.org). Manuscripts must be accompanied by a cover letter, signed by all authors, with a statement that the manuscript has been read and approved by them, and not published, submitted or accepted elsewhere. Manuscripts, which are accepted for publication in the Journal, become the property of the Journal, and may not be published anywhere else without written permission from the publisher.

Serbian Journal of Experimental and Clinical Research is owned and published by Faculty of Medical Sciences, University of Kragujevac. However, Editors have full academic freedom and authority for determining the content of the journal, according to their scientific, professional and ethical judgment. Editorial policy and decision making follow procedures which are endeavoring to ensure scientific credibility of published content, confidentiality and integrity of auth ors, reviewers, and review process, protection of patients' rights to privacy and disclosing of conflict of interests. For difficulties which might appear in the Journal content such as errors in published articles or scientific concerns about research findings, appropriate handling is provided. The requirements for the content, which appears on the Journal internet site or Supplements, are, in general, the same as for the master version. Advertising which appears in the Journal or its internet site is not allowed to influence editorial decisions.

MANUSCRIPT

Manuscripts for Serbian Journal of Experimental and Clinical Research are available for submission through the Editorial Manager System http://www.editorialmanager.com/sjecr/.

For papers that are accepted, Serbian Journal of Experimental and Clinical Research obligatory requires authors to provide an identical, electronic copy in appropriate textual and graphic format.

The manuscript of original, scinetific articles should be arranged as following: Title page, Abstract, Introduction, Patients and methods/Material and methods, Results, Discussion, Acknowledgements, References, Tables, Figure legends and Figures. The sections of other papers should be arranged according to the type of the article.

Each manuscript component (The Title page, etc.) should begins on a separate page. All pages should be numbered consecutively beginning with the title page.

All measurements, except blood pressure, should be reported in the System International (SI) units and, if necessary, in conventional units, too (in parentheses). Generic names should be used for drugs. Brand names may be inserted in parentheses.

Authors are advised to retain extra copies of the manuscript. Serbian Journal of Experimental and Clinical Research is not responsible for the loss of manuscripts in the mail.

TITLE PAGE

The Title page contains the title, full names of all the authors, names and full location of the department and institution where work was performed, abbreviations used, and the name of corresponding author.

The title of the article should be concise but informative, and include animal species if appropriate. A subtitle could be added if necessary.

A list of abbreviations used in the paper, if any, should be included. The abbreviations should be listed alphabetically, and followed by an explanation of what they stand for. In general, the use of abbreviations is discouraged unless they are essential for improving the readability of the text.

The name, telephone number, fax number, and exact postal address of the author to whom communications and reprints should be sent are typed et the end of the title page.

ABSTRACT

An abstract of less than 250 words should concisely state the objective, findings, and conclusions of the studies described in the manuscript. The abstract does not contain abbreviations, footnotes or references.

Below the abstract, 3 to 8 keywords or short phrases are provided for indexing purposes. The use of words from Medline thesaurus is recommended.

INTRODUCTION

The introduction is concise, and states the reason and specific purpose of the study.

PATIENTS AND METHODS/MATERIAL AND METHODS

The selection of patients or experimental animals, including controls, should be described. Patients' names and hospital numbers are not used.

Methods should be described in sufficient detail to permit evaluation and duplication of the work by other investigators.

When reporting experiments on human subjects, it should be indicated whether the procedures followed were in accordance with ethical standards of the Committee on human experimentation (or Ethics Committee) of the institution in which they were done and in accordance with the Helsinki Declaration. Hazardous procedures or chemicals, if used, should be described in details, including the safety precautions observed. When appropriate, a statement should be included verifying that the care of laboratory animals followed accepted standards.

Statistical methods used should be outlined.

RESULTS

Results should be clear and concise, and include a minimum number of tables and figures necessary for proper presentation.

DISCUSSION

An exhaustive review of literature is not necessary. The major findings should be discussed in relation to other published work. Attempts should be made to explain differences between the results of the present study and those of the others. The hypothesis and speculative statements should be clearly identified. The Discussion section should not be a restatement of results, and new results should not be introduced in the discussion.

ACKNOWLEDGMENTS

This section gives possibility to list all persons who contributed to the work or prepared the manuscript, but did not meet the criteria for authorship. Financial and material support, if existed, could be also emphasized in this section.

ARTICLE REFERENCES – VANCOUVER STYLE

References should be identified in the text by Arabic numerals in parentheses. They should be num be red consecutively, as they appeared in the text. Personal communications and un published observations should not be cited in the reference list, but may be mentioned in the text in parentheses. Abbreviations of journals should conform to those in Index Serbian Journal of Experimental and Clinical Research. The style and punctuation should con form to the Serbian Journal of Experimental and Clinical Research style requirements. The following are examples:

Vancouver style

Article in a journal:

You CH, Lee KY, Chey WY, Menguy R. Electrogastrographic study of patients with unexplained nausea, bloating and vomiting. Gastroenterology 1980;79:311-4; DOI:10.2478/s11533-007-0023-3.

Book:

Eisen HN. Immunology: an introduction to molecular and cellular principles of the immune response. 5th ed. New York: Harper and Row; 1974.

1. Introduction

This document describes standards for preparing the references in the APA style. The following sections give detailed instructions on citing books, journal articles, newspaper articles, conference papers, theses, webpages and others.

Please provide all the required elements in the references to your paper. Please pay particular attention to spelling, capitalization and punctuation. Accuracy and completeness of references are the responsibilities of the author. Before submitting your article, please ensure you have checked your paper for any relevant references you may have missed.

A complete reference should give the reader enough information to find the relevant article. And most importantly, complete and correct references may allow automatic creation of active links by the MetaPress technology that we use for making the electronic version of our journal. Active reference linking is regarded as the greatest benefit of electronic publishing and it adds a lot of value to your publication.

2.Book

a. Book (one author)

Format:

Author. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Baxter, R. (1982). *Exactly Solvable Models in Statistical Mechanics.* New York: Academic Press.

b. Book (two or more authors)

Format:

Author1, Author2 & Author3. (Year of publication). *Book title*. Place of publication: Publisher.

Example:

Kleiner, F.S., Mamiya C.J. & Tansey R.G. (2001). *Gardner's art through the ages* (11th ed.). Fort Worth, USA: Harcourt College Publishers.

c. Book chapter or article in an edited book

Format:

Author(s) of chapter. (Year of publication). Chapter title. In Editors of the book (Eds.), *Book title* (Chapter page range). Place of publication: Publisher.

Example:

Roll, W.P. (1976). ESP and memory. In J.M.O. Wheatley & H.L. Edge (Eds.), *Philosophical dimensions of parapsychology* (pp. 154-184). Springfield, IL: American Psychiatric Press.

d. Proceedings from a conference

Format:

Author(s). (Year of publication). Title. In Conference name, Date (Page range). Place of publication: Publisher.

Example:

Field, G. (2001). Rethinking reference rethought. In Revelling in Reference: Reference and Information Services Section Symposium, 12-14 October 2001 (pp. 59-64). Melbourne, Victoria, Australia: Australian Library and Information Association.

e. ebook

Format:

Author(s). (Year of publication). *Title*. Publisher. Retrieving date, http address. DOI.

Example:

Johnson, A. (2000). *Abstract Computing Machines*. Springer Berlin Heidelberg. Retrieved March 30, 2006, from SpringerLink http://springerlink.com/content/w25154. DOI: 10.1007/b138965.

f. Thesis

Format:

Author(s). (Year of publication). *Title*. Information, Place of publication.

Example:

Begg, M. M. (2001). *Dairy farm women in the Waikato 1946-1996: Fifty years of social and structural change*. Unpublished doctoral dissertation, University of Waikato, Hamilton, New Zealand.

g. Report

Format:

Author(s). (Year of publication). *Title*. Place of publication: Publisher. (Report number)

Example:

Osgood, D. W., & Wilson, J. K. (1990). *Covariation of adolescent health problems*. Lincoln: University of Nebraska. (NTIS No. PB 91-154 377/AS)

h. Government publication

Format:

Institution name. (Year of publication). *Title.* Place of publication: Publisher.

Example:

Ministerial Council on Drug Strategy. (1997). *The national drug strategy: Mapping the future*. Canberra: Australian Government Publishing Service.

TABLES

Tables should be typed on separate sheets with table numbers (Arabic) and title above the table and explanatory notes, if any, below the table.

FIGURES AND FIGURE LEGENDS

All illustrations (photographs, graphs, diagrams) will be considered as figures, and numbered consecutively in Arabic numerals. The number of figures included should be the least required to convey the message of the paper, and no figure should duplicate the data presented in the tables or text. Figures should not have titles. Letters, numerals and symbols must be clear, in proportion to each other, and large enough to be readable when reduced for publication. Figures should be submitted as near to their printed size as possible. Figures are reproduced in one of the following width sizes: 8 cm, 12 cm or 17 cm, and with a maximal length of 20 cm. Legends for figures should be given on separate pages.

If magnification is significant (photomicrographs) it should be indicated by a calibration bar on the print, not by a magnification factor in the figure legend. The length of the bar should be indicated on the figure or in the figure legend.

Two complete sets of high quality unmounted glossy prints should be submitted in two separate envelopes, and shielded by an appropriate cardboard. The backs of single or grouped illustrations (plates) should bear the first authors last name, figure number, and an arrow indicating the top. This information should be penciled in lightly or placed on a typed self-adhesive label in order to prevent marking the front surface of the illustration.

Photographs of identifiable patients must be accompanied by written permission from the patient.

For figures published previously the original source should be acknowledged, and written permission from the copyright holder to reproduce it submitted.

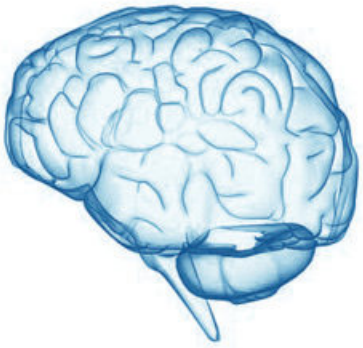
Color prints are available by request at the authors expense.

LETTERS TO THE EDITOR

Both letters concerning and those not concerning the articles that have been published in Serbian Journal of Experimental and Clinical Research will be considered for publication. They may contain one table or figure and up to five references.

PROOFS

All manuscripts will be carefully revised by the publisher desk editor. Only in case of extensive corrections will the manuscript be returned to the authors for final approval. In order to speed up publication no proof will be sent to the authors, but will be read by the editor and the desk editor.



CIP - Каталогизација у публикацији
Народна библиотека Србије, Београд

61

SERBIAN Journal of Experimental and Clinical Research
editor-in-chief Vladimir Jakovljević.
- Vol. 9, N° 1 (April 2008) -
- Kragujevac (Svetozara Markovića 69) :
Medical Faculty, 2008 - (Kragujevac : Medical Faculty). - 29 cm

Je nastavak: Medicus (Kragujevac) = ISSN 1450-7994
ISSN 1820-8665 = Serbian Journal of
Experimental and Clinical Research
COBISS.SR-ID 149695244

Serbian Journal of Experimental and Clinical Research

2000

FACULTY OF MEDICAL SCIENCES
Svetozara Markovica 69, 34000 Kragujevac, SERBIA
P.O. Box 124
Tel. +381 (0)34 30 68 00 • Tfx. +381 (0)34 30 68 00 ext. 112
e-mail: sjecr@medf.kg.ac.rs

www.medf.kg.ac.rs