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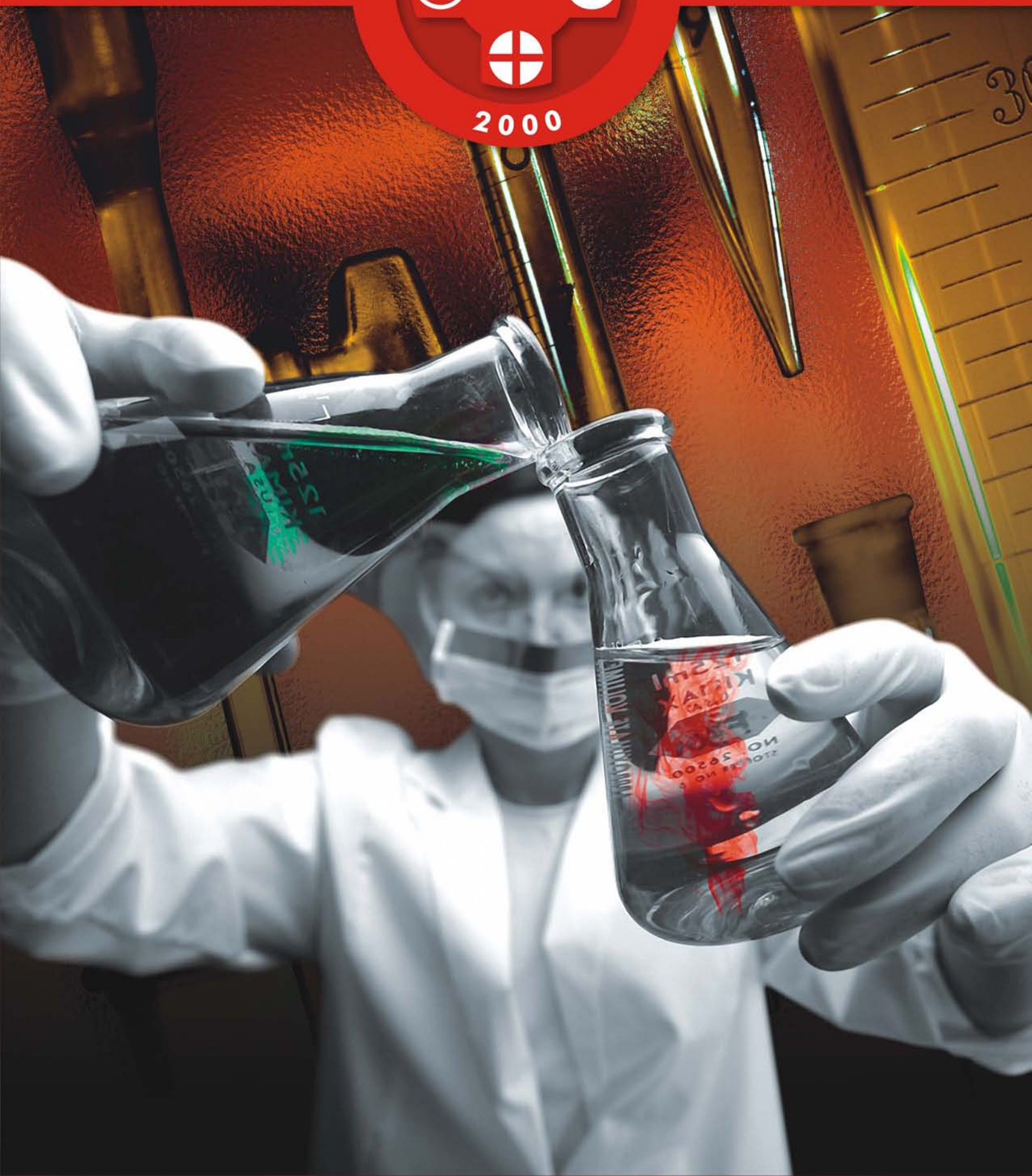

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Table Of Contents

LABORATORY DIAGNOSIS OF HEPARIN INDUCED THROMBOCYTOPENIA

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ABSTRACT

The laboratory diagnosis of heparin-induced thrombocytopenia (HIT) is based on the identification of antibodies against the complex between heparin and platelet factor 4 (PF4) by functional and/or immunological methods. These methods are complicated, time- and labour intensive. Therefore an introduction of a rapid, easy to perform method for the identification of circulating HPA is of high importance. ID-PaGIA heparin/PF4 assay may be an appropriate candidate. It should be emphasized that the diagnosis of HIT remains the primarily clinical, using pre-test clinical probability scoring system (4T's) while laboratory results should be considered as an additional tool in confirming or ruling out clinical diagnosis. An algorithm for HIT diagnosis with combination of clinical and laboratory findings is presented.

Key words: *Heparin induced thrombocytopenia (HIT), ID-PaGIA, 4T's score.*

Heparin induced thrombocytopenia (HIT) is a severe, potentially limb- and life-threatening immune-mediated adverse drug reaction to unfractioned heparin and/or low molecular weight heparin which may occur in up to 3% of treated patients. In spite of thrombocytopenia, (most commonly a 50% fall in platelet count, beginning most usually between 5-14 days after initial exposure to any dose or type of heparin) [1-3], bleeding is uncommon, while thromboembolic complications are the main clinical problem in patients with HIT.

HIT is caused by the formation of antibodies that activate platelets following heparin administration, with a complex of heparin and platelet factor 4 (PF4) as a principal antigen. HIT is a clinicopathological syndrome and the diagnosis of HIT remains primarily clinical, while it should be supported by confirmatory laboratory testing.

Thrombocytopenia in HIT is generally modest, with platelet counts of 50-70 X109/L, while severe thrombocytopenia (< 10X109/L) is unusual. After an initial exposure to heparin, platelet decrease starts on day 4 or 5 after the formation of IgG antibodies. However, patients exposed to heparin within the last 3 months (100 days), develop abrupt thrombocytopenia within 24 hours after re-exposure to heparin. A gradual decline in platelet count beginning on the first day of heparin therapy, with a decrease in platelet count to 50% of baseline over the first 4-5 days of therapy, is less consistent with a HIT diagnosis [4].

The American College of Chest Physicians (ACCP) recommends that platelet count should be monitored every 2 to 3 days (every second day for postoperative prophylaxis with unfractioned heparin (UFH)) with beginning on the 4th day after the initiation of heparin therapy, until the therapy is discontinued or until the 14th day of heparin exposure in the following clinical settings: any therapeutic dosing of UFH and low molecular weight heparin (LMWH), surgical and medical prophylaxis with UFH and LMWH, UFH prophylaxis in obstetrical patients and in postoperative patients receiving prophylactic-dose LMWH, or intravascular catheter UFH "flushes". Monitoring is not recommended for prophylactic medical/obstetrical use of LMWH, or for medical patients receiving UFH flushes. If there is a history of heparin exposure within the last 100 days, a platelet count is recommended within 24 hours of heparin re-exposure [5].

HIT is one of the most common adverse drug reactions, but there are many other, more common causes for thrombocytopenia, especially in the setting of severe illness and major surgery [6]. Clinically, HIT is a diagnosis made after the exclusion of more likely causes of thrombocytopenia. Recovery of platelet counts after cessation of heparin therapy is also significant for diagnosis of HIT.

Occurrence of new thrombosis during the administration of heparin anticoagulation should raise significant suspicion for the diagnosis of HIT, while development of allergic reaction to the heparin treatment could also be of importance.

Assessment of clinical aspects of a suspected case of HIT utilizing the scoring system may guide the appropriate use and interpretation of antibody testing. Therefore the pre-test clinical probability scoring system (4T's) seems to be a valuable tool for HIT diagnosis (Table 1) [7].

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	Points (0, 1 or 2 for each category: maximum possible score = 8)		
	2	***1***	***0***
Thrombocytopenia	> 50% fall or platelet nadir 20–100 $g \cdot L^{-1}$	30–50% fall or platelet nadir 10–19 $g \cdot L^{-1}$	Fall < 30% or platelet nadir < 10 $g \cdot L^{-1}$
Timing of platelet count fall or other sequelae	Clear onset between day 5–day 10; or less than 1 day (if heparin exposure within past 100 days)	Consistent with immunization but not clear (e.g., missing platelet counts) or onset of thrombocytopenia after day 10	Platelet count fall too early (without recent heparin exposure)
Thrombosis or other sequelae (e.g. skin lesions)	New thrombosis; skin necrosis; post heparin bolus acute systemic reaction	Progressive or recurrent thrombosis; erythematosus skin lesions; suspected thrombosis not yet proven	None
Other cause for thrombocytopenia not evident	No other cause for platelet count fall is evident	Possible other cause is evident	Definite other cause is present
Pre-test clinical probability score: 6–8 = high; 4–5 = intermediate; 0–3 = low			

Table 1: Pre-clinical test probability (4T's) score [7]

LABORATORY DIAGNOSIS OF HIT

Detection of HIT antibodies is necessary, but not sufficient, for the diagnosis of HIT. Laboratory diagnosis of HIT relies on the detection of antibodies against heparin/PF4 complex in plasma or serum with functional and/or immunological methods.

Immunological methods, such as an ELISA are available in most clinical laboratories and they detect circulating IgG, IgA and IgM antibodies against heparin/PF4 complex. The most usual threshold for a positive test result in the available commercial kits is an optical density (OD) of 0.400. Since only some antibodies (e.g. IgG) are of clinical importance, sensitivity to all three subclasses of antibodies decreases assay specificity (50-93%) [8, 9]. Increasing cut-off values of OD (to 1 or 1.4) could improve assay specificity [10]. At the same time, the sensitivity of this method is high (>97%) with the negative predictive value of > 95% [11, 12]. Therefore negative results obtained with ELISA may be used for ruling out a HIT diagnosis, while positive results should be combined with clinical findings and/or functional assay. The specificity of this method could be improved using IgG specific ELISA assay which is currently under evaluation in several laboratories. We have shown potentially better specificity of this assay in comparison with standard ELISA in recent pilot study [13].

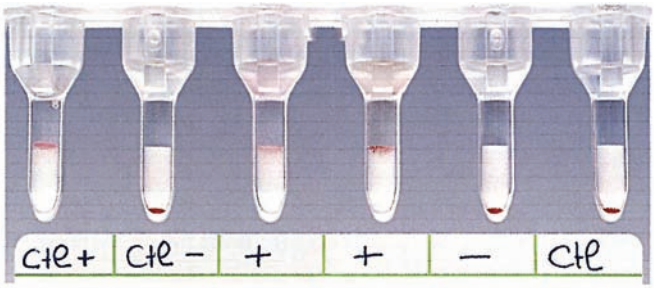
Functional methods measure the activation of platelets of healthy donors after the addition of patients' plasma and heparin (conc. 0.1 – 1 IU/mL). Heparin induced platelet aggregation assay performed in donor's platelet rich plasma is most commonly used in clinical laboratories. The sensitivity of this method is good (>90%) while the specificity for detecting clinically relevant (pathogenic) antibodies is higher than with the commercially available antigen assay (77-97%) as a consequence of the fact it exclusively detects platelet-activating antibodies of immunoglobulin IgG class (only IgG antibodies can activate platelets via their Fc or IgG-receptors) [11]. Another assay, the serotonin release assay (SRA), utilizes donor platelets in which serotonin is radiolabeled by C14. The addition of heparin results in platelet activation and release of radiolabeled serotonin which is detected. This assay has the advantage of proving that an anti-PF4-heparin antibody in the patient's sample can actually stimulate platelet activation and therefore this assay expresses the highest specificity and is considered the "gold standard" [1, 8]. However, the use of radioactive isotopes, special equipment requirements and lack of experienced staff limit this method to the very few highly specialized reference laboratories.

No single assay has 100% sensitivity and specificity and therefore it seems that the optimal laboratory diagnostic approach is a combination of both functional and antigen assays. However, both ELISA and functional assays are complicated, time consuming and labor intensive and could be performed in most laboratories only during daily working hours. Therefore, one rapid, easy to perform method for the detection of circulated heparin/PF4 antibodies is highly desirable because the decision about the possible interruption of heparin treatment should not be delayed.

ID-PAGIA HEPARIN/PF4 ASSAY

ID-PaGIA heparin/PF4 (DiaMed) is a rapid particle gel immunoassay that detects IgG, A and M specific to heparin/PF4 complexes. The principle behind this method is widely used in blood group serology determination in transfusion medicine. Briefly, 10 µl of plasma are placed in the reaction chamber of the test ID-card (containing a buffered sephacryl gel matrix) followed by 50 µl of polymer particles (red high density polystyrene beads coated with heparin/PF4 complexes which serve as the solid-phase in a particle agglutination assay). After 5 minutes of incubation at room temperature the ID-card is centrifuged for 10 minutes in the appropriate ID-centrifuge.

Results are presented qualitatively (positive/negative) and can be read directly without the employment of additional equipment. When anti-heparin-PF4 antibodies are present in the plasma, the particles are cross-linked and remain at the top of the gel chamber (positive results). If there is no significant level of anti-heparin/PF4 antibodies, all the particles sink to the bottom of the gel chamber (negative result).



Negative ELISA is used to adequately and safely rule out HIT diagnosis. A very good overall agreement between ELISA and PaGIA (86%) was described in one previously published study [14]. We have recently observed similar results (90% of overall agreement) [15]. However, one out of 82 samples was considered falsely negative on ID-PaGIA since both ELISA and platelet aggregation assay were positive and clinical criteria for HIT were fulfilled. It was also previously calculated that there was 16% probability for HIT diagnosis in high risk patients even if the ID-PaGIA assay was negative [16]. This is further supported by findings of potentially lower sensitivity of ID-PaGIA in comparison to ELISA (94 vs 100%) observed in one study [17]. Lack of information about the clinical status in patients from that study rendered the relevance of those findings uncertain. Nevertheless, it seems that the interpretation of negative results should be cautious in patients with a high pre-test clinical probability score.

In spite of this the use of PaGIA as a routine 24-hour available screening test for the diagnosis of HIT seems to be justified. ID-PaGIA may replace the standard ELISA and offers a possibility to rule out HIT diagnosis within minutes after the request for laboratory testing in the majority of patients. That would decrease the amount of unnecessary switches of heparin to other anticoagulant treatments which are both costly and may increase the risk of bleeding complications.

RISK FOR OVER-DIAGNOSIS OF HIT

PF4-heparin antibodies could be detected using described laboratory assays in many different patient populations. Up to 50% of all individuals undergoing cardiac surgery develop HIT antibodies, but only a small percentage actually manifests clinical HIT [9]. Up to 3% of patients receiving UFH for medical prophylaxis will have a positive ELISA for heparin/PF4 antibodies, but only 0.5% of them develop thrombocytopenia [18]. The use of heparin for chronic hemodialysis is associated with a 12% prevalence of heparin/PF4 antibodies, but nearly all cases of HIT during hemodialysis occur within the first 3-4 weeks of initiation [19]. This suggests that HIT is unlikely to develop in the setting of chronic heparin exposure.

Those data suggest that HIT diagnosis should not be based on laboratory tests. Laboratory results should be considered only as an additional tool in confirming or ruling out clinical diagnosis. The pre-test clinical probability scoring system (4T's) should therefore be combined with laboratory results in establishing HIT diagnosis.

SUMMARY

Focusing on the laboratory diagnosis of HIT, according to our own experience at Karolinska University Hospital, discussions and preliminary recommendations from Nordic Laboratory Group on HIT, ECAT pilot survey and study on HIT diagnostics, as well as most available literature data, it seems that:

- 4T's pre-test clinical probability score must be used in the HIT diagnostic algorithm;
- High specificity and negative predictive value (>95%) justify ELISA as the most appropriate first line assay for laboratory investigation of HIT (standard Ig G, A, M ELISA may be replaced with IgG specific ELISA according to preliminary data but the absence of false negative results should be confirmed in larger studies).
- In spite of better specificity, due to lower sensitivity and different pitfalls (complexity, different heparin concentration, source of donor platelets) it seems that heparin induced platelet aggregation could not be used in a laboratory diagnosis of HIT alone. Performed in combination with ELISA, this assay should be interpreted together with the pre-test clinical probability score (4Ts's). Platelet rich plasma (PRP) or "washed" platelets collected from at least 3 donors should be used in aggregation assay, while the concentration of heparin should be 0.1 – 1 IU/mL. Positive samples may be further tested with high heparin concentration (10 or 100 IU/mL) to avoid non-immunological reactivity to heparin.
- The use of a rapid, 24 hour available screening method may be beneficial in routine work. ID-PaGIA may be a good candidate and it expresses slightly lower sensitivity and potentially better specificity than ELISA. However, the interpretation of negative results should be cautious, especially in patients with a high pre-test clinical probability score.

There is no conflict of interest to declare - I do not have any relation with Diamed - a manufacturer of ID-PaGIA.

ALGORITHM FOR HIT DIAGNOSIS:

- HIT diagnosis may be ruled-out with >95% probability if ID-PaGIA/ELISA are negative. A negative ID-PaGIA test should be completed with ELISA in patients with a high pre-test clinical probability score.

- Positive ID-PaGIA/ELISA indicates HIT diagnosis in patients with high pre-test clinical probability score. Further testing is not necessary.
- Positive ID-PaGIA/ELISA should combine with the functional method – heparin induced platelet aggregation in patients with low and intermediate pre-test clinical probability score:
  - o Positive heparin induced platelet aggregation test increases the probability for HIT diagnosis in those patients. HIT is probable.
  - o Negative heparin induced platelet aggregation test increases the probability for ruling out HIT diagnosis. HIT is however still possible in patients with an intermediate pre-test clinical probability score.

LITERATURE

1. Warkentin TE & Grienacher A, Heparin-induced thrombocytopenia: recognition, treatment, and prevention: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy. Chest. 2004; 126(3 Suppl): 311S-337S.
2. Arepally GM & Ortel TL. Clinical practice. Heparin-induced thrombocytopenia. N Engl J Med. 2006; 355: 809-17.
3. Warkentin TE. Heparin-induced thrombocytopenia. Hematol Oncol Clin North Am. 2007; 21: 589-607.
4. Hassell K. Heparin-induced thrombocytopenia: diagnosis and management. Thromb Res. 2008; 123 (Suppl 1): S16-21.
5. Warkentin TE, Greinacher A, Koster A, Lincoff AM; American College of Chest Physicians. Treatment and prevention of heparin-induced thrombocytopenia: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). Chest. 2008; 133(6 Suppl): 340S-380S.
6. Selleng K, Warkentin TE, Greinacher A, Heparin-induced thrombocytopenia in intensive care patients, Crit Care Med. 2007; 34: 1165–76.
7. Warkentin TE. Heparin-induced thrombocytopenia: pathogenesis and management. Br J Haematol. 2003; 121: 535-5.
8. Warkentin TE, Sheppard JA, Moore JC, Moore KM, Sigouin CS, Kelton JG. Laboratory testing for the antibodies that cause heparin-induced thrombocytopenia: how much class do we need? J Lab Clin Med. 2005; 146: 341-6.
9. Warkentin TE, Sheppard JA, Horsewood P, Simpson PJ, Moore JC, Kelton JG. Impact of the patient population on the risk for heparin-induced thrombocytopenia. Blood. 2000; 96: 1703-8.
10. Warkentin TE, Sheppard JI, Moore JC, Sigouin CS, Kelton JG. Quantitative interpretation of optical density measurements using PF4-dependent enzyme-immunoassays. J Thromb Haemost. 2008; 6: 1304-12.
11. Pouplard C, Amiral J, Borg JY, Laporte-Simitsidis S, Delahousse B, Gruel Y. Decision analysis for use of platelet aggregation test, carbon 14-serotonin release assay, and heparin-platelet factor 4 enzyme-linked immunosorbent assay for diagnosis of heparin-induced thrombocytopenia. Am J Clin Pathol. 1999; 111: 700-6.
12. Lo GK, Juhl D, Warkentin TE, Sigouin CS, Eichler P, Greinacher A. Evaluation of pretest clinical score (4 T's) for the diagnosis of heparin-induced thrombocytopenia in two clinical settings. J Thromb Haemost. 2006; 4: 759-65.
13. Antovic JP, Fareed J, Hoppensteadt D, Prechel M, Norberg E, Sten-Linder M. Comparison of immunological assays for detection of heparin/PF4 antibodies in HIT patients. Abstract XXII ISTH Congress, Boston, USA, 2009.
14. Meyer O, Salama A, Pittet N, Schwind P. Rapid detection of heparin-induced platelet antibodies with particle gel immunoassay (ID-HPF4). Lancet. 1999; 354(9189):1525-6.
15. Antovic JP, Norberg EM, Sten-Linder M. Evaluation of "Particle Gel Immuno Assay (ID-PaGIA Heparin/PF4)" - a new rapid method for identification of heparin/PF4 antibodies. Abstract Book of Swedish Doctor Society Annual Meeting, Gothenburg 2008.
16. Pouplard C, Gueret P, Fouassier M, Ternisien C, Trossaert M, Regina S, Gruel Y. Prospective evaluation of the '4Ts' score and particle gel immunoassay specific to heparin/PF4 for the diagnosis of heparin-induced thrombocytopenia. J Thromb Haemost. 2007; 5: 1373-9.
17. Eichler P, Raschke R, Lubenow N, Meyer O, Schwind P, Greinacher A. The new ID-heparin/PF4 antibody test for rapid detection of heparin-induced antibodies in comparison with functional and antigenic assays. Br J Haematol. 2002; 116: 887-91.
18. Giolami B, Prandoni P, Stefani PM, Tanduo C, Sabbion P, Eichler P et al. The incidence of heparin-induced thrombocytopenia in hospitalized medical patients treated with subcutaneous unfractionated heparin: a prospective cohort study, Blood. 2003; 101: 2955–9.
19. Luzzatto G, Bertoli M, Cella G, Fabris F, Zaia B, Girolami A. Platelet count, anti-heparin platelet factor 4 antibodies and tissue factor pathway inhibitor plasma antigen level in chronic dialysis, Thromb Res. 1998; 89: 115–22.

FOLIC ACID EFFECTS ON THE ACETYL CHOLINESTERASE ACTIVITIES IN DIFFERENT TISSUES OF A RAT

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UTICAJ FOLNE KISELINE NA AKTIVNOST ACETILHOLINESTERAZE U RAZLIČITIM TKIVIMA PACOVA

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ABSTRACT

Various studies have shown that the serum concentration of folic acid is inversely related to the serum homocysteine concentration. In addition, data suggest that increases in serum homocysteine levels decrease the activity of acetyl cholinesterase (AChE). The aim of this study was to investigate the effects of acute folic acid treatment on AChE activity in the brain, heart and the blood of a rat. We used male Wistar rats, with a body mass of 250-300 g divided into two categories: a control group given placebo (1 ml 0.9% NaCl, i.p. n1=6) and an experimental group treated with folic acid (1 ml 0.011 μmol/g per body mass, pH 7.4, i.p., n2=6). Sixty minutes after the treatment, the rats were decapitated. The brain and the heart were removed, and blood was taken. The brain and heart were homogenised in phosphate buffer pH 8 (1 ml buffer/20 mg tissue). The homogenised tissues and blood were used as a substrate for the spectrophotometric analysis, and enzyme activity was measured in vitro by the Ellman method. Rats treated with folic acid showed significantly reduced AChE activity in the brain and blood compared to the control group (38% and 82%, respectively). We did not observe a significant difference between the enzyme activity in the blood of treated versus untreated rats. In conclusion, this study shows that acute treatment with folic acid decreases the AChE activity in the brain and heart of rats without affecting the AChE activity in the blood.

Key words: *acetyl cholinesterase, folic acid, specific enzyme activity*

SAŽETAK

Istraživanja su pokazala da je koncentracija folne kiseline obrnuto proporcionalna koncentraciji homocisteina u serumu. Takođe, postoje podaci da povećanje koncentracije homocisteina u serumu smanjuje aktivnost acetilholinesteraze (AChE). Cilj ovog istraživanja bio je da se ispitaju efekti akutne aplikacije folne kiseline na aktivnost enzima AChE u tkivu mozga, srca i krvi pacova. U eksperimentu su korišćeni pacovi mužjaci soja Wistar, telesne mase 250-300 g, podeljeni u dve grupe: kontrolna grupa kojoj je aplikovan placebo (1 ml 0,9% NaCl, i.p., n1=6) i eksperimentalna grupa kojoj je aplikovana folna kiselina (1 ml 0,011 μmol/g telesne mase pH 7,4, i.p., n2=6). Šezdeset minuta posle tretmana životinje su žrtvovane dekapitovanjem. Izolovani su mozak i srce, i uzeta je krv. Izvršena je homogenizacija moždanog i srčanog tkiva u fosfatnom puferu pH 8,0 (1 ml pufera/20 mg tkiva). Homogenizovana tkiva i krv korišćeni su kao supstrat za spektrofotometrijsku analizu. Aktivnost enzima u ispitivanim tkivima merena je in vitro metodom po Ellmanu. Rezultati su pokazali su da je aktivnost AChE u homogenatima tkiva mozga i srca pacova tretiranih folnom kiselinom značajno manja od aktivnosti enzima u homogenatima tkiva kontrolne grupe (za 38% i 82%, respektivno). Nije utvrđena značajna razlika u aktivnosti enzima u krvi životinja koje su tretirane folnom kiselinom u odnosu na kontrolnu grupu. Zaključeno je da aplikacija folne kiseline smanjuje aktivnost AChE u mozgu i srcu pacova, dok ne utiče na aktivnost AChE u krvi pacova.

Ključne reči: *acetilholinesteraza, folna kiselina, specifična enzimska aktivnost*

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INTRODUCTION

Acetyl cholinesterase (AChE, EC 3.1.1.7) is a serine hydrolase enzyme with the primary function of hydrolysing the neurotransmitter acetylcholine (1, 2, 3). According to the number of catalysed substrate molecules per unit time, AChE is the most efficient enzyme from the serine hydrolase group and one the most powerful enzymes overall (1, 2). AChE is a highly polymorphic enzyme (1, 3). The significant polymorphism occurs as a result of a difference and interaction between catalysed subunits forming the enzyme and the mechanism of "anchoring" enzymes into the cell membrane and extracellular matrix (1, 6). Apart from its main function of hydrolysing acetylcholine, AChE plays major roles in cellular adhesion, all phases of the intrauterine development of the central nervous system, induction and promotion of axonal growth, synaptic maturation and chematopoese (3, 6, 7).

Folic acid is responsible for numerous biochemical reactions in mammals such as nucleic acid synthesis, other reactions of transmetalation, homocysteine metabolism and enzymatic regeneration of tetrahydrobiprotein (8). Recent studies have revealed a significant role for folic acid in regulating peripheral and coronary blood flow (8, 9). Insufficient intake of folic acid is often correlated with slower growth, anaemia, weight loss, indigestion and even behavioural disruptions (10). Furthermore, studies indicate a significant inverse correlation between folic acid intake and the concentration of homocysteine in serum (11, 12, 13, 15). In addition, some groups have reported that an elevated homocysteine level decreases the activity of AChE (13, 14). It is well known that increased serum homocysteine concentration represents a risk factor for developing cardiovascular diseases, mental retardation and disturbance of the intrauterine development of the nervous system (12, 13, 15).

Given the clinical importance of increased serum homocysteine concentration and its known reciprocal relationship folic acid, we thought it important to investigate the influence of folic acid on AChE activity in the brain, heart and blood of rats.

MATERIALS AND METHODS

a) Experimental procedure

This experiment was performed on Wistar albino male rats weighing between 250-300 g. The animals were kept in a standard laboratory environment (temperature 22±1 °C, humidity 50%, 12:12 h cycle light: darkness, with the beginning of the light cycle at 9:00 h) with free access to water and food. We observed all regulations regarding animal care prescribed by the Regulations on Experimental Animals of the Faculty of Medicine, Belgrade, and permission of the Ethical Committee on experimental animals was obtained. The rats were divided into two groups: the control group was administered placebo (1 ml 0.9% NaCl, i.p., n1=6), and the experimental group was given folic acid (1 ml 0.011 μmol/g per body mass pH 7.4, i.p., n2=6). Since the folic acid was given intraperitoneally, it was dissolved in physiological saline (sodium chloride) (0.9% NaCl) and buffered to pH 7.4. Sixty minutes after treatment, the animals were decapitated. The brain and the heart were removed while the blood was taken and stored in test tubes coated with heparin. Brain and heart tissues were homogenised in phosphate buffer pH 8.0 (1 ml buffer/20 mg tissue). The homogenised tissues and blood were subjected to spectrophotometric analysis.

b) Defining the AChE activity

The AChE activity of each tissue was assessed in vitro by the Ellman method (16). This method is based on the hydrolysis reaction of the coloured reagent 5.5'-dithiobis (2-nitrobenzoic acid), (DNTB) with the thiocholine substrate, acetylcholine-Iodide (AChI), producing 5-thio-2 -nitrobenzoic. The resultant yellow colour intensity is proportional to the activity of AChE. The suitable quantity of homogenate (20 μl brain homogenate in 600 μl of phosphate buffer pH 8.0, 40 μl heart homogenate in 580 μl of phosphate buffer pH 8.0, 50 μl of heparinised blood diluted in physiological saline (sodium chloride), in proportion 1:100 in 570 μl of phosphate buffer, pH 8.0), was pre-incubated for ten minutes at 37°C. After the preincubation, 20 μl of the coloured reagent DTNB was added in addition to 10 μl of the AchI substrate. We measured the change in absorbance at 412 nm over three minutes using a spectrophotometer (Gilford Instrument, model 250). The blind test had all the essay components for monitoring AChE activities except for the homogenate of the tested tissues. The measurements performed in duplicate, and the specific enzyme activity of AChE in the brain and heart was formulated as ΔA/(min x mg tissue) and ΔA/(min x μL blood) in the blood.

c) Statistics processing

The data processing was performed by single factorial analysis of the variable, while intergroup comparisons were made using the Bonferroni test. The values were presented as X±SD, and values with $p<0.05$ were considered statistically significant.

d) Materials

The following substances were used to perform the experiments: folic acid, acetylthocoline-Iodide (AChI), 5.5'-dithiobis (2-nitrobenzoic acid) (DNTB), (all from Sigma Chemicals Co., USA) All the substances used in the experiment were of pro analysis quality.

RESULTS

The final results show a statistically significant difference in the activity levels of AChE between the tested tissues of the control group animals (brain vs. heart $p<0.01$, brain vs. blood $p<0.01$, heart vs. blood $p<0.05$). The highest enzyme activity was recorded in the brain tissue homogenate (0.194±0.020), then heart tissue ho-

mogenate (0.110±0.028), and the lowest was recorded in the blood (0.067±0.030). In the experimental group, we observed changes in the AChE activity in all of the tested tissues compared to the control group. The measured AChE activity in the brain tissue homogenate of the experimental group (0.121±0.041) was significantly lower than the AChE activity in the brain tissue homogenate of the control group; (0.194±0.020) ($p<0.05$) (Table 1, Diagram 1). The treatment reduced the AChE activity in the brain tissue by 38%. The heart tissue homogenate in the rats treated with folic acid showed significantly lower specific AChE activity (0.020±0.010) than the heart tissue homogenate of the control group (0.110±0.028) ($p<0.01$) (Table 1, Diagram 1). The parenteral application of folic acid reduced the AChE activity in the heart tissue by 82%. Although the AChE activity in the blood of the experimental animals (0.090±0.027) was higher than the activity in the blood of control animals (0.067±0.030) by 34%, this difference was not statistically significant (Table 1, Diagram 1).

Within the group of animals treated with folic acid, we observed a statistically significant difference in the specific enzyme activity of the brain and the heart tissue homogenates (brain vs. heart $p<0.01$) and the heart homogenate and blood (heart vs. blood $p<0.01$). The difference in the AChE activity between the brain and blood was not statistically significant. The highest significant enzyme activity was recorded in the brain tissue homogenate (0.121±0.041), then the blood (0.090±0.027), and the lowest activity was recorded in the heart tissue homogenate (0.020±0.010).

TESTED GROUPS	BRAIN ΔA (MIN X MG TISSUE)	HEART ΔA (MIN X MG TISSUE)	BLOOD ΔA (MIN X ML BLOOD)
Control	0.194 ± 0.020	0.110 ± 0.028	0.067 ± 0.030
Experimental (folic acid)	0.121 ± 0.041	0.020 ± 0.010	0.090 ± 0.027

Table 1: Median values and standard deviations (X±SD) of AchE activity in the tested tissues

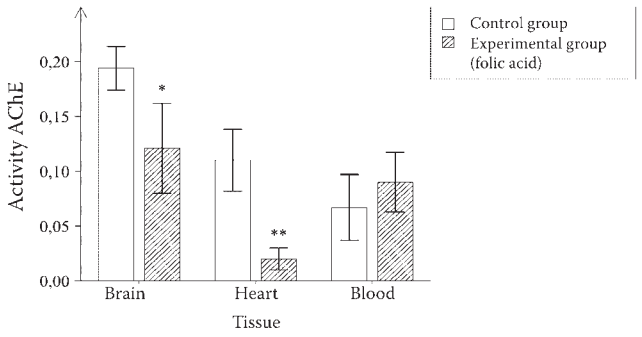

Diagram 1: AchE activity levels in the tested tissues of the control and folic acid treatment group (* $p<0,05$, ** $p<0,01$)

DISCUSSION

This study investigated the effects of the acute administration of folic acid on AChE activity in the brain, heart and blood of rats. The results show that the examined tissues show significant differences in baseline enzyme activity in the control animals. The AChE activity was highest in the brain, then heart and finally in the blood, which confirms previously published results (17). Namely, the group of authors had shown in their researches the approximately identical ratio of the specific AChE activities in the tissues of the brain, heart and blood of younger rats (17). The results also show that folic acid significantly reduces AChE activity in the brain (by 38%) and heart (by 82%) of rats, but it does not significantly affect AChE activity in the blood. Little is understood about the effects of folic acid on AChE activity. Nevertheless, numerous studies have shown that folic acid intake reduces the concentration of homocysteine in serum (11, 12, 13, 15). Some authors have also reported increasing the concentration of homocysteine in serum leads to reduced AChE activity (13, 14). In vitro analyses (13) and animal experiments (14) support these findings.

On the basis of the data we possess, we may assume that folic acid increases the AChE activity. Our study shows that folic acid reduces the AChE enzyme activity in the brain and the heart of rats, but it does not significantly affect the activity in the blood. The results lead to the conclusion that folic acid does not affect AChE activity in the tissues by the proposed homocysteine mechanism. Further research is required to clarify the mechanism of this process.

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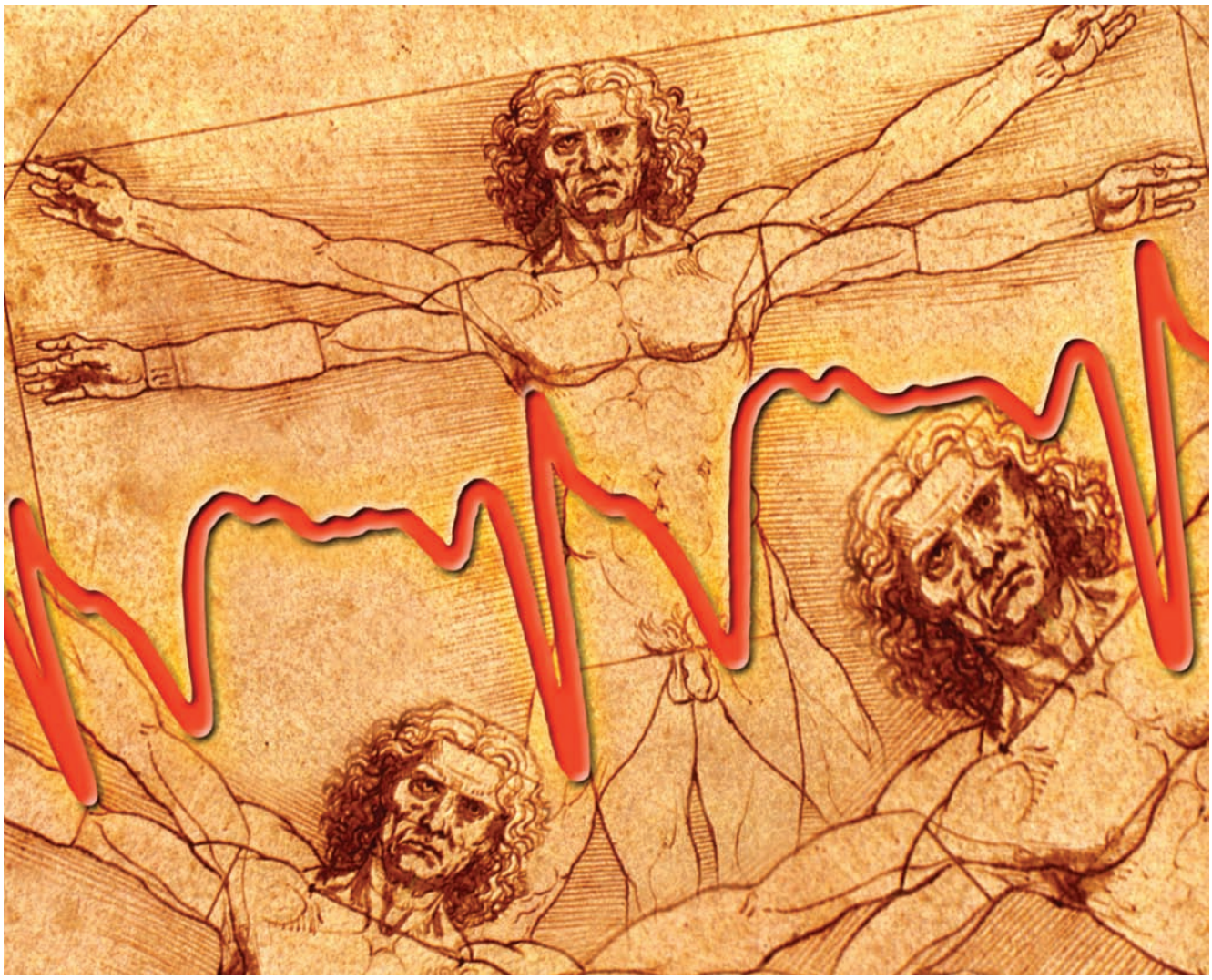
REFERENCES

1. Small D, Michaelson S, Sberna G. Non-classical actions of cholinesterases: Role in cellular differentiation, tumorigenesis and Alzheimer's disease. Neurochemistry International 1996; 28(5-6):453-483.
2. Silman I, Sussman J. Acetylcholinesterase: How is structure related to function? Chemico-Biological Interactions 2008; 175(1-3):3-10.
3. Massoulié J, Perrier N, Noureddine H, Liang D, Bon S. Old and new questions about cholinesterases. Chemico-Biological Interactions 2008; 175(1-3):30-44.
4. Tsim K, Choi R, Xie H et al. Transcriptional control of different subunits of AChE in muscles: signals triggered by the motor nerve-derived factors. Chemico-Biological Interactions 2008; 175(1-3):58-63.
5. Rotundo R, Ruiz C, Marrero E et al. Assembly and regulation of acetylcholinesterase at vertebral neuromuscular junction. Chemico-Biological Interactions 2008; 175(1-3):26-29.
6. Silman I, Sussman J. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. Current Opinion in Pharmacology 2005; 5(3):293-302.

7. Slotkin T. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicology and Applied Pharmacology 2004; 198(2):132-151.
8. Tawakol A, Migrino R, Aziz K et al. High-dose folic acid acutely improves coronary vasodilator function-in patients with coronary artery disease. JACC 2005; 45(10):1580-1584.
9. Djurić D, Vušanović A, Jakovljević V. The effects of folic acid and nitric oxide synthase inhibition on coronary flow and oxidative stress markers in isolated rat heart. Molecular and Cellular Biochemistry 2007; 300:177–183.
10. Patterson D. Folate metabolism and the risk of Down syndrome. Down's syndrome, Research and Practice 2008; 12(2):93-97.
11. Anglister L, Etlin A, Finkel E, Durrant A, Lev-Tov A. The effect of folic acid supplementation on plasma homocysteine in an elderly population. Chemico-Biological Interactions 2008; 175(1-3):92-100.
12. Strandhagen E, Landaas S, Thelle D. Folic acid supplement decreases the homocysteine increasing effect of filtered coffee. A randomised placebo-controlled study. European Journal of Clinical Nutrition 2003; 57(11):1411-1417.
13. Schulpis K, Kalimeris K, Bakogiannis C, Tsakiris T, Tsakiris S. The effect of in vitro homocystinuria on the suckling rat hippocampal acetylcholinesterase. Metabolic Brain Disease 2006; 21(1):21-28.
14. Stefanello F, Zugno A. Wannmacher C et al. Homocysteine inhibits butyrylcholinesterase activity in rat serum. Metabolic Brain Disease 2003; 18(3):187-194.
15. Lamers Y, Prinz-Langenohl R, Moser R, Pietrzik K. Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. The American Journal of Clinical Nutrition 2004; 79(3):473-478.
16. Ellman G, Courtney K, Andreas V, Featherstone R. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 1961; 7:88-90.
17. Carr R, Chambers H, Guarisco J, Richardson J, Tang J, Chambers J. Effects of repeated oral ostnatal exposure to chlorpyrifos on open-field behavior in juvenile rats. The Journal of Toxicological Sciences 2001; 59(2):260-267.



THE STUDY OF E-CADHERIN EXPRESSION IN GLOTTIC LARYNGEAL SQUAMOUS CELL CARCINOMA

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EKSPRESIJA E-KADHERINA KOD SKVAMOCELULARNIH KARCINOMA GLOTIČNE REGIJE LARINKSA

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ABSTRACT

Background: E-cadherin is a 120 kDa transmembrane protein that is thought to play an important role in malignant progression of tumours and in tumour differentiation. A reduced or absent expression of E-cadherin has been observed in several carcinomas, including squamous cell carcinoma of the head and neck.

Objective: The aim of this study was to analyse the clinicopathologic significance of E-cadherin expression in squamous cell carcinomas with a primary location in the glottic region of the larynx.

Materials and methods: E-cadherin expression was determined by immunohistochemistry in paraffin-embedded tissue specimens from 40 patients with squamous cell carcinoma of the glottic larynx. A staining score was given based on the percentage of cells stained (0–100%). All stained cells were considered positive regardless of the intensity of the staining. Using the mean expression of E-cadherin as a cut-off, 17 (42.5%) tumours were classified into the "high E-cadherin" group and 23 (57.5%) into the "low E-cadherin" group.

Results: E-cadherin expression varied greatly among the tissue samples, with scores ranging from 2 to 72 (median 23). The mean expression score for E-cadherin was 27.35 (standard deviation [SD]=20.15). Decreased E-cadherin expression was significantly correlated with more aggressive tumours, including tumours staged as T3 or T4 (p = 0.038) and those with advanced clinical stage (TNM stage III and IV) (p = 0.010). The results of a stepwise logistic regression analysis showed that only the presence of lymph node metastasis was an independent predictor for tumour recurrence (p=0.019). A Cox proportional hazards model confirmed that the presence of cervical lymph node metastases (P=0.003) and age ≤ 59 years (P=0.006) were statistically significant independent predictors of a reduced disease-specific survival.

Conclusion: Expression of E-cadherin may be useful to identify patients with aggressive disease, allowing more effective treatment strategies to be implemented.

SAŽETAK

"Ekspresija E-kadherina kod skvamocelularnih karcinoma glotične regije larinksa".

Uvod: E-kadherin je transmembranski protein molekularne mase 120 kDa koji ima važnu ulogu u progresiji i diferencijaciji tumora. Odsustvo ili smanjena ekspresija E-kadherina nađena je za veliki broj neoplazmi uključujući i karcinome glave i vrata.

Cilj istraživanja bio je da se analizira kliničko-patološki značaj ekspresije E- kadherina u pacijenata sa planocelularnim karcinomom grkljana lokalizovanim u glotisu.

Materijal i metode: Ekspresija E- kadherina analizirana je imunohistohemijski u 40 pacijenata sa glotisnim karcinomom grkljana. Rezultat imunohistohemijske ekspresije E-kadherina pretstavljao je procenat obojenih ćelija (0–100%). Sve obojene ćelije su uključene u brojanje bez obzira na intenzitet. U odnosu na srednju vrijednost ekspresije ispitanici su podijeljeni u dvije grupe. U 17 (42.5%) slučajeva se radilo o visokoj ekspresiji (procenat obojenih ćelija veći od srednje vrijednosti) a 23 (57.5%) pacijenta su imali nisku ekspresiju E- kadherina (procenat manji od srednje vrijednosti).

Rezultati: Ekspresija E- kadherina u posmatranom materijalu varirala je 2 do 72 (mediana 23). Srednja vrijednost ekspresije E- kadherina iznosila je 27.35 (standardna devijacija [SD]= 20.15). Značajno slabija ekspresija E-kadherina nađena je u pacijenata sa lokalno proširenim tumorom (kategorija T3 i T4) (χ2-test p= 0.038) kao i u pacijenata sa uznapredovalom bolešću (TNM stadijum III i IV) (χ2-test p= 0.010). Multivarijantnom logističkom regresionom analizom dobili smo da je prisustvo metastaza na vratu jedini nezavisni prediktor relapsa bolesti (p=0.019). Rezultati Cox-ove regresione analize pokazuju da su nezavisni prediktori kraćeg preživljavanja bez bolesti prisustvo metastaza na vratu (P=0.003) i starosna dob ≤ 59 godina (P=0.006).

Zaključak: Određivanje ekspresije E- kadherina moglo bi da pomogne u otkrivanju pacijenata sa agresivnim tipom bolesti što bi vodilo određivanju optimalnog

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Larger studies are required to confirm the role of E-cadherin expression in predicting the behaviour of laryngeal squamous cell carcinomas.

Key words: *Laryngeal carcinomas, Cell adhesion molecule, E-cadherin, Immunohistochemistry, Head and neck.*

načina liječenja. Uloga E-kadherina u procjeni biološkog ponašanja karcinoma larinksa treba da bude potvrđena dodatnim istraživanjima.

Ključne reči: *karcinom larinksa, ćelijski adhezioni molekul, E-kadherin, imunohistohemija, glava i vrat*

INTRODUCTION

The development of cancer involves multiple coordinated cellular processes. Identification of the molecular mechanisms involved in laryngeal cancer progression will contribute to a better understanding of its biological behaviour. Multiple steps are required to induce tumour invasion and metastasis, including the expression of a variety of gene products that include adhesion molecules. Weakening of cell-cell and cell-extracellular matrix adhesions is obviously imperative for tumour cells to metastasise. Several families of cell adhesion molecules have been described. These include cadherins, integrins, adhesion molecules belonging to the immunoglobulin superfamily, selectins, and CD44. The cadherins are a group of calcium-dependent adhesion molecules that mediate homotypic cell-cell interactions, although heterotypic binding between different cadherin molecules is possible. They have an extracellular domain (N-terminal) that is implicated in homophilic binding and a cytoplasmic tail (C-terminal) that interacts with cytoskeletal proteins via intracellular proteins termed catenins (α, β, γ), which form the E-cadherin-catenin complex (1). There are four cadherin subclasses (classical cadherins, classical-related cadherins, desmosomal cadherins and modified cadherins). Among these adhesion molecules, epithelial cadherin (E-cadherin) is the most important, since it is expressed in all adult human epithelial tissues. A reduced or absent expression, or an abnormal location, of the E-cadherin/catenin complex has been observed in several carcinomas, including malignant tumours of the female genital tract (2), stomach (3), nasopharynx (4), bladder (5), prostate (6), lung (7), colon (8), breast (9) and squamous cell carcinomas of the head and neck (10-12).

In the present study, we used immunohistochemistry to examine the expression of E-cadherin in invasive glottic laryngeal squamous cell carcinomas, and we correlated our results with clinicopathological parameters.

MATERIALS AND METHODS

Patients

Forty patients with squamous cell carcinoma of the glottic larynx were selected from the pathological files of the Clinic for Otorhinolaryngology and Maxillofacial Surgery of the Clinical Center of Montenegro in Podgorica. All selected patients underwent complete resection as primary treatment in the period from 2001 to 2008. All patients had a single primary tumour, none had undergone treatment prior to surgery, and all had microscopically clear surgical margins. None of the patients was thought to have had distant metastases at the time of surgery. The clinical information, including sex, age, histologic grade, primary tumour (T) classification, nodal (N) status, TNM stage, and oncological outcome were obtained retrospectively from clinical records. Pathological staging was determined according to the 6th TNM Classification of Malignant Tumours of the International Union Against Cancer. Twenty patients had early cancer (Stage I or II) and 20 had advanced cancer (Stage III or IV). Treatment decision-making was based on clinical stage and on the presence or absence of lymph node metastases at the time of diagnosis. Partial laryngectomy was performed in 26 patients, and total laryngectomy in 14 patients. Nine patients underwent a neck dissection operation simultaneously to the primary tumour removal, and lymph node metastases were present in four cases. Eight patients underwent postoperative radiotherapy. Mean follow-up time (calculated in months from treatment completion to the last otolaryngological control) was 20.5 months (range 6-60 months). In the analysis of the clinical data, we defined poor oncological outcome as either recurrence of local disease or occurrence of metastasis after treatment. Clinicopathologic characteristics of the selected patients are shown in Table 1.

Immunohistochemistry

Forty specimens of formalin-fixed, paraffin-embedded tissue blocks were cut into 3-mm sections by a microtome. All specimens included samples originated from complete resection material. The slides were dewaxed, hydrated, and washed with TRIS-buffered saline. This process was followed by microwave treatment for 20 min in citrate buffer (pH= 6.0) to retrieve the antigens present. After blocking endogenous peroxidase activity in water with 3% H202 for 30 min, the tissue sections were incubated with anti-E-cadherin antibody (Clone NCH-38 diluted 1:50, DAKO, Denmark) for 30 min and then anti-mouse antibody for another 30 min. Immunodetection was performed with the Envision system, DAKO Autostainer, model VL1. Diaminobenzidine was applied for 10 min as a chromogen. The slides were then counterstained with hematoxylin. Appropriate positive and negative controls were included in all reactions.

Evaluation of E-cadherin expression

The slides were viewed randomly and without any clinical data by one of the authors. The staining was predominantly membranous with some cytoplasmatic staining also

	No. of patients	%
Sex		
Male	29	72.5
Female	11	27.5
T stage		
T1	12	30
T2	10	25
T3	16	40
T4	2	5
N stage		
N0	36	90
N1	3	7.5
N2	1	2.5
TNM stage		
I	12	30
II	8	20
III	18	45
IV	2	5
Histological grading		
G1	23	57.5
G2	17	42.5
Loco-regional recurrence		
L-R rec. no	32	80
L-R rec. yes	8	20

Table 1: Clinicopathologic characteristics of 40 patients with glottic squamous cell carcinoma

present. A staining score was given based on the percentage of cells stained (0–100%). All stained cells were considered positive regardless of the intensity of the staining.

STATISTICAL ANALYSIS

The correlations between the clinicopathologic parameters and the expression of E-cadherin were evaluated using the chi-square ($\chi 2$) test, the Fisher exact test and Kruskal-Wallis test. The role of each possible prognostic factor (univariate analysis) and the joint effect of all these factors (multivariate analysis) was explored using a multivariate logistic regression analysis. Disease-free survival analysis was based on the Kaplan-Meier method, and statistical significance was assessed by the log-rank test. To determine the effect of distinct prognostic factors on survival, a multivariate analysis was performed according to the Cox regression model. A p value less than 0.05 was considered to be significant in all statistical analyses. All statistical analyses were conducted with SPSS 13.0 (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, USA).

RESULTS

Expression of E-cadherin was evaluated in 40 glottic laryngeal squamous cell carcinomas (29 male and 11 female). The age of the patients at diagnosis ranged from 44 to 81 years with a mean age of 63.2 years. Twenty patients had early cancer (Stage I or II) and 20 had advanced cancer (Stage III or IV).

Table 2: The correlation of E-cadherin expression with clinicopathologic parameters in patients with glottic squamous cell carcinoma

Variables	Number of patients	E-cadherin expression[a]		
		Low	High	p value[b]
Sex				1.0[c]
Male	29	17	12	
Female	11	7	4	
Age (years)				.792
? 59	16	10	6	
> 59	24	14	10	
T classification				.038
T1 and T2	22	10	12	
T3 and T4	18	14	4	
N status				.136
N0	36	20	16	
N+	4	4	0	
TNM stage				.010
I and II	20	8	12	
III and IV	20	16	4	
Histologic grade				.601
G1	23	13	10	
G2 and G3	17	11	6	
Loco-regional recurrence				.114
L-R rec. no	32	17	15	
L-R rec. yes	8	7	1	

a Low E-cadherin expression was below 27.35, High E-cadherin expression was above 27.35
b Chi-square ($\chi 2$) test or Fisher exact test

E-cadherin expression was associated with the cell membrane and varied greatly among tissue samples, with scores ranging from 2 to 72 (median 23). The mean expression score of E-cadherin in the considered glottic squamous cell carcinomas was 27.35 (standard deviation [SD] = 20.15). Using the mean expression of E-cadherin as a cut-off, 17 (42.5%) tumours were classified into the "high E-cadherin" group and the other 23 (57.5%) tumours were classified into the "low E-cadherin" group.

The correlations of E-cadherin expression with clinicopathologic parameters is summarised in Table 2. Decreased E-cadherin expression was significantly correlated with more aggressive tumours, including T3-T4-staged tumours (p= 0.038) and those with advanced clinical stage (TNM stage III and IV) (p= 0.010). There was no significant correlation between the expression of E-cadherin and age or sex. No relationship was observed between E-cadherin expression and histopathological differentiation (p= 0.601). Also, the Fisher exact test did not show any statistically significant difference in E-cadherin expression between pN+ and pN0/cN0 malignancies (p= 0.136).

The mean expression of E-cadherin was 38.5 (SD 22.49) in pT1 carcinomas, 24.5 (SD 19.54) in pT2 carcinomas, 22.75 (SD 16.92) in pT3 carcinomas, and 11.5 (SD 12.02) in pT4 carcinomas. We performed a non-parametric test for trends across order groups (a modified Kruskal-Wallis test) to identify differences in E-cadherin between pT stages, but no significant differences were shown (p= 0.140). The mean E-cadherin expression was 38.5 (SD 22.45) in stage I carcinomas, 25.63 (SD 21.97) in stage II carcinomas, 22.44 (SD 15.94) in stage III carcinomas and 11.5 (SD 12.02) in stage IV carcinomas. The differences in expression between the different TNM stages were not statistically significant (p= 0.140).

Eight of 40 patients with laryngeal squamous cell carcinomas developed loco-regional recurrence (3 local recurrences, 5 recurrences in the neck lymph nodes; mean expression of E-cadherin 17.63; median 18.5; SD 9.46). In the group without loco-regional recurrence, mean expression of E-cadherin was 29.78 (median 26; SD 21.45). Correlations of E-cadherin expression with tumour recurrence were not statistically significant (p=0.114).

The results of a stepwise logistic regression analysis showed that only the presence of lymph node metastasis was an independent predictor for tumour recurrence when grouping local and regional recurrences (odds ratio 18.6; p= 0.019; 95% CI, 1.601-216.056). There was a decline in disease-free survival associated with a decreased E-cadherin expression, but this was not significant (log-rank test p= 0.111). The presence of lymph node metastasis at the time of diagnosis (log-rank p= 0.002), age ≤ 59 years (log-rank p=0.003), and female gender (log-rank p=0.025) were all associated with worse disease-free survival. The results of a multivariate Cox proportional hazards model confirmed that the presence of cervical lymph node metastases (P=0.003) and age ≤ 59 years (P=0.006) were statistically significant independent predictors of a reduced disease-specific survival.

DISCUSSION

The presence of lymph node metastases is the single most adverse independent prognostic factor in head and neck squamous cell carcinoma (13). The preoperative detection of lymph node metastases is crucial to the effective treatment of these patients. Diagnostic techniques including computed tomography, magnetic resonance imaging, ultrasonography, positron emission tomography, and ultrasound-guided fine-needle aspiration biopsy have reached a sensitivity of more than 80% in detecting metastases (14), but they have the fundamental limitation that the metastases need to have a size of at least several millimetres to be detected. Thus, the ability to identify molecular markers from a primary tumour biopsy sample that can predict cervical lymph node metastases would enable the selection of patients at risk for lymph node metastasis. However, since invasion and metastasis are very complicated multistep processes, it is likely that more than one marker will be needed to assess an individual patient's risk of nodal metastases.

The process of metastasis is a cascade of linked sequential steps involving multiple host-tumour interactions. The suppression of cell-cell adhesiveness is accepted to have an important role in facilitating the dissemination of tumour cells from the primary site and the establishment of metastases (15). Among the several families of adhesion molecules, E-cadherin has been suggested to play a role in the process of nodal metastasis. E-cadherin, a calcium-dependent membrane protein, is essential for the formation of adheren junctions between cells (16). Catenins are involved in the regulation of cadherin function. Both β and γ catenins bind directly to the cytoplasmic portion of E-cadherin. α catenin plays a critical role in the transmembrane anchorage of the cadherins, and deletion of α catenin results in a non-adhesive cadherin/catenin phenotype. Downregulation of E-cadherin reduces cell-cell adhesion, reduces gap-junction mediated communication, and prevents terminal differentiation of cells, thus maintaining the ability to proliferate (17). The loss of E-cadherin expression in tumour tissue may lead to a more aggressive phenotype because neoplastic cells have a greater tendency to spread to adjacent tissues and lymph nodes. Abnormal expression of E-cadherin has been correlated in several human carcinomas with pathological characteristics of the tumour, such as tumour stage, invasiveness, lymph node involvement and distant metastases (18-21). Moreover, reduced expression of E-cadherin has been correlated with clinical variables, such as disease relapse and disease-free survival (22-24). Additionally, a negative correlation between E-cadherin expression and tumour differentiation has been observed. Well- or moderately-differentiated cancers preserve E-cadherin, while poorly differentiated ones express it poorly or not at all (10).

Studies of E-cadherin expression in squamous cell carcinomas of the head and neck have failed to provide a clear picture for its role in these tumours. Schipper et al. (12)

observed that E-cadherin expression decreased with loss of differentiation in primary carcinomas and that lymph node metastases expressed a lower level of the protein, suggesting an important role of cadherin loss in the metastatic process. In 1996, Franchi and colleagues (25) observed that low expression of E-cadherin in laryngeal squamous cell carcinomas significantly correlated with the presence of occult nodal metastases. Simionescu et al. (26) studied 42 cases of oral squamous cell carcinomas at different sites (tongue, lips, palate, and gums) and different grades of differentiation and investigated the immunoexpression of adhesion molecules. The study indicated a decreasing degree of immunostaining for E-cadherin parallel with decreases in oral squamous cell carcinoma differentiation grade. The loss of cell adhesion correlated to a decrease in E-cadherin expression, suggesting that E-cadherin may be a good prognostic predictor in oral squamous cell carcinoma evolution. Eriksen et al. (27) found that E-cadherin was strongly correlated with histopathological features associated with well-differentiated tumours and can be considered as a marker of differentiation in squamous cell carcinomas of the head and neck. Mattijssen et al. (11) also studied a group of 50 patients with head and neck squamous cell carcinoma. A relation between high levels of membrane-associated E-cadherin expression and favourable outcomes was found, although it did not reflect an absence of regional lymph node metastases. Liu et al. (28) studied markers associated with tumour invasion and metastasis in 59 patients with laryngeal and hypopharyngeal squamous cell carcinoma with node metastases. No relationship was found between the immunopositivity of cancer cells for E-cadherin and the presence of lymph node metastasis. Takes et al. (29) examined histological features and biological markers in 31 patients with laryngeal carcinomas. Of all markers investigated immunohistochemically, E-cadherin was not relevant to the prediction of lymph node metastasis.

In this study, we analysed the clinicopathologic significance of E-cadherin expression among 40 patients with laryngeal squamous cell carcinomas primary localised in glottic region. Our study found that lower expression of E-cadherin was significantly associated with a more aggressive tumour phenotype, including T3-T4 tumours and those with advanced clinical stage (TNM stage III and IV). There was no significant correlation between the expression of E-cadherin and age or sex. Generally, E-cadherin expression was found to be high in well differentiated cancers, but it was reduced in undifferentiated cancers (10-12, 25). Our results show a general, but not significant, decline in E-cadherin expression with increasing dedifferentiation of the tumour, a finding that is in line with those of Rodrigo et al. (30). Some studies have demonstrated a correlation between reduced E-cadherin expression and nodal metastases (12, 25), whereas others have failed to show this relationship (10, 29). In our study, the Fisher exact test did not show any statistically significant difference in expression of E-cadherin between pN+ and pN0/cN0 malignancies. The lack of statistical association between the nodal status and expression of E-cadherin was possibly due to the limited size of our preliminary series. In addition, we failed to find any significant association of E-cadherin expression with tumour recurrence.

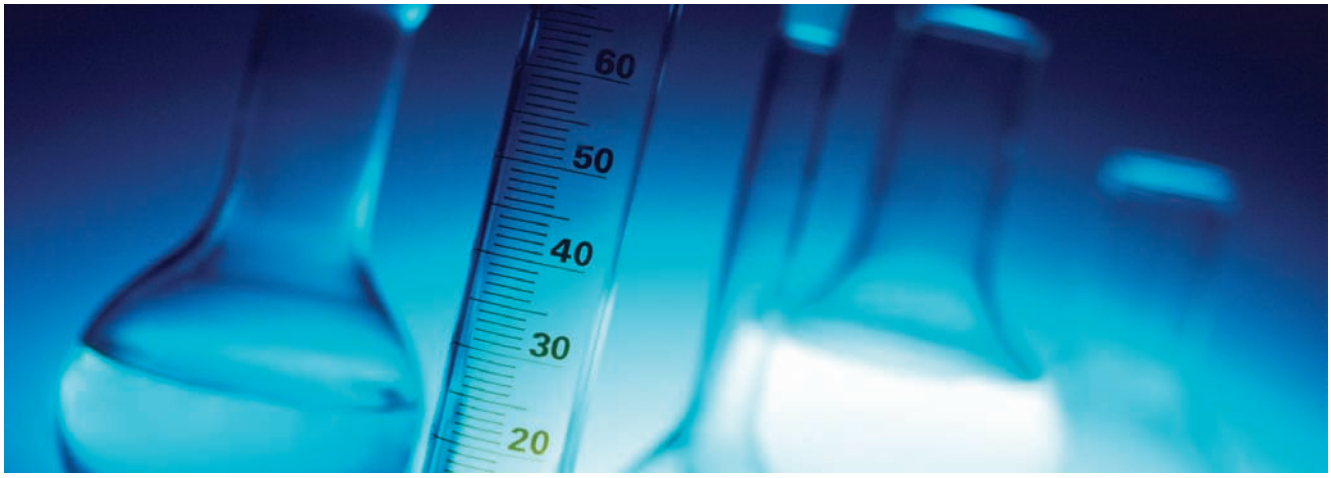
CONCLUSION

Decreased expression of E-cadherin in primary glottic laryngeal squamous cell carcinomas correlated significantly with advanced T status and TNM stage. The results of the present study suggest that expression of E-cadherin may be useful to identify patients with more aggressive disease, allowing more effective treatment strategies to be implemented. Larger studies are required to confirm the role of E-cadherin expression in predicting the behaviour of laryngeal squamous cell carcinomas.

REFERENCES

1. Shimoyama Y, Hirohashi S, Hirano S, et al. Cadherin cell-adhesion molecules in human epithelial tissues and carcinomas. Cancer Res 1989; 49: 2128–33.
2. Veatch AL, Carson LF, Ramakrishnan S. Differential expression of the cell-cell adhesion molecule E-cadherin in ascites and solid human ovarian tumor cells. Int J Cancer 1994; 58: 393-9.
3. Chen HC, Chu RY, Hsu PN, et al. Loss of E-cadherin expression correlates with poor differentiation and invasion into adjacent organs in gastric adenocarcinomas. Cancer Lett 2003; 201 : 97-106.
4. Li Z, Ren Y, Lin SX, Liang YJ, Liang HZ. Association of E-cadherin and beta-catenin with metastasis in nasopharyngeal carcinoma. Chin Med J 2004; 117: 1232-9.
5. Sun W, Herrera GA. E-cadherin expression in urothelial carcinoma in situ, superficial papillary transitional cell carcinoma, and invasive transitional cell carcinoma. Hum Pathol 2002; 33: 996-1000.
6. Koksal IT, Ozcan F, Kilicaslan I, Tefekli A. Expression of E-cadherin in prostate cancer in formalin-fixed, paraffin-embedded tissues: correlation with pathological features. Pathology 2002; 34: 233-8.
7. Bohm M, Totzeek B, Birchmeier W, Wieland I. Differences of E-cadherin epression levels and patterns in primary and metastatic human lung cancer. Clin Exp Metastasis 1994; 12: 55-62.
8. Kanazawa T, Watanabe T, Kazama S, Tada T, Koketsu S, Nagawa H. Poorly differentiated adenocarcinoma and mucinous carcinoma of the colon and rectum show higher rates of loss of heterozygosity and loss of E-cadherin expression due to methylation of promoter region. Int J Cancer 2002; 102: 225-9.
9. Sarrio D, Perez-Mies B, Hardisson D, et al. Cytoplasmic localization of p120ctn and E-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions. Oncogene 2004; 23: 3272-83.

10. Bowie GL, Caslin AW, Roland NJ, Field JKM, Jones AS, Kinsella AR. Expression of the cell-cell adhesion molecule E-cadherin in squamous cell carcinoma of the head and neck. Clin Otolaryngol 1993; 18: 196-201.
11. Mattijssen V, Peters HM, Schalkwijk L, et al. E-cadherin expression in head and neck squamous cell carcinoma is associated with clinical outcome. Int J Cancer 1993; 55:580-5.
12. Schipper JH, Frixen UH, Behrens J, Unger A, Jahnke K, Birchmeier W. E-cadherin expression in squamous cell carcinomas of head and neck. Inverse correlation with tumor differentiation and lymph node metastasis. Cancer Res 1991; 51: 6328-37.
13. Forastiere A, Koch W, Trotti A, Sidransky D. Head and neck cancer. N Engl J Med 2001; 345: 1890-900.
14. van den Brekel MWM, Castelijns JA, Snow GB. Diagnostic evaluation of the neck. Otolaryngol Clin North Am 1998; 31: 601-19.
15. Wijnhoven BPL, Dinjens WNM, Pignatelli M. E-cadherin-catenin cell-cell adhesion complex and human cancer. Br J Surg 2000; 87: 992-1005.
16. Guilford P. E-cadherin downregulation in cancer: fuel on the fire? Mol Med Today 1999; 5: 172–7.
17. Jongen WM, Fitzgerald DJ, Asamoto M, et al. Regulation of connexin 43-mediated gap junctional intercellular communication by Ca2+ in mouse epidermal cells is controlled by E-cadherin. J Cell Biol 1991; 114: 545–55.
18. Bukholm IK, Nesland JM, Karesen R, Jacobsen U, Borresen-Dale AL. E-cadherin and alpha-, beta-, and gamma-catenin protein expression in relation to metastasis in human breast carcinoma. J Pathol 1998; 185: 262-6.
19. Pignatelli M, Ansari TW, Gunter P, et al. Loss of membranous E-cadherin expression in pancreatic cancer: correlation with lymph node metastasis, high grade, and advanced stage. J Pathol 1994; 174: 243–8.
20. Shun CT, Wu MS, Lin JT, et al. An immunohistochemical study of E-cadherin expression with correlations to clinicopathological features in gastric cancer. Hepatogastroenterology 1998; 45: 944-9.
21. De Marzo AM, Knudsen B, Chan-Tack K, Epstein JI. E-cadherin expression as a marker of tumor aggressiveness in routinely processed radical prostatectomy specimens. Urology 1999; 53: 707-13.
22. Tamura S, Shiozaki H, Miyata M, et al. Decreased E-cadherin expression is associated with haematogenous recurrence and poor prognosis in patients with squamous cell carcinoma of the oesophagus. Br J Surg 1996; 83: 1608-14.
23. Gabbert HE, Mueller W, Schneiders A, et al. Prognostic value of E-cadherin expression in 413 gastric carcinomas. Int J Cancer 1996; 69: 184-9.
24. Charpin C, Garcia S, Bonnier P, et al. Reduced E-cadherin immunohistochemical expression in node-negative breast carcinomas correlates with 10-year survival. Am J Clin Pathol 1998; 109: 431-8.
25. Franchi A, Gallo O, Boddi V, Santucci M. Prediction of occult metastases in laryngeal carcinoma: role of proliferating cell nuclear antigen, MIB-1, and E-cadherin immunohistochemical determination. Clin Cancer Res 1996; 2: 1801-8.
26. Simionescu C, Mărgăritescu C, Surpățeanu M, et al. The study of E-cadherine and CD44 immunoexpression in oral squamous cell carcinoma. Rom J Morphol Embryol 2008; 49(2): 189-93.
27. Eriksen JG, Steiniche T, Søgaard H, Overgaard J. Expression of integrins and E-cadherin in squamous cell carcinomas of the head and neck. APMIS 2004; 112 (9): 560-8.
28. Liu M, Lawson G, Delos M, et al. Prognostic value of cell proliferation markers, tumor suppressor proteins and cell adhesion molecules in primary squamous cell carcinoma of the larynx and hypopharynx. Eur Arch Otorhinolaryngol 2003; 260: 28-34.
29. Takes RP, Baatenburg de Jong RJ, Schuuring E, et al. Markers for assesment of nodal metastasis in laryngeal carcinoma. Arch Otolaryngol Head Neck Surg 1997; 123: 412-9.
30. Rodrigo JP, Domínguez F, Alvarez C, Manrique C, Herrero A, Suárez C. Expression of E-cadherin in squamous cell carcinomas of the supraglottic larynx with correlations to clinicopathological features. Eur J Cancer 2002; 38(8): 1059-64.



NON-TRADITIONAL RISK FACTORS FOR DEVELOPMENT OF CARDIOVASCULAR COMPLICATIONS IN HAEMODIALYSIS PATIENTS

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NETRADICIONALNI FAKTORI RIZIKA ZA RAZVOJ KARDIOVASKULARNIH KOMPLIKACIJA KOD BOLESNIKA NA HEMODIJALIZI

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ABSTRACT

Cardiovascular diseases are a leading cause of death in patients treated with haemodialysis. These patients are exposed to traditional and non-traditional risk factors for cardiovascular complications. Non-traditional risk factors are consequences of uremic milieu, but these can also be linked to the technique of dialysis itself. Non-traditional risk factors include oxidative stress, microinflammation, malnutrition, secondary hyperparathyroidism, anaemia, hyperhomocysteinemia, retention of sodium and water and increase of blood flow through the vascular access for haemodialysis. These risk factors are implicated in left ventricle hypertrophy and accelerate atherosclerosis. In addition, they increase cardiovascular morbidity and mortality in these patients. Aggressive cardiovascular risk factor modification can significantly improve cardiovascular outcome in patients treated with haemodialysis.

Key words: *cardiovascular risk factors, microinflammation, malnutrition, haemodialysis.*

SAŽETAK

Kardiovaskularne bolesti su vodeći uzrok smrti bolesnika koji se leče hemodijalizom. Ovi bolesnici su izloženi tradicionalnim i netradicionalnim faktorim rizika za razvoj kardiovaskularnih komplikacija. Netradicionalni faktori rizika su posledica uremijskog miljea, a mogu biti povezani i sa samom tehnikom dijalize. U netradicionalne faktore rizika spadaju mikroinflamacija, hiperhomocisteinemija, povećana koncentracija asimetričnog dimetilarginina, oksidativni stres, malnutricija, sekundarni hiperparatireoidizam, anemija, retencija Na+ i H2O i povećan protok krvi kroz vaskularni pristup za hemodijalizu. Pomenuti faktori rizika uzrokuju hipertrofiju leve komore i ubrzanu aterosklerozu, a to za posledicu ima povećan kardiovaskularni morbiditet i mortalitet ovih bolesnika. Uticajem na faktore rizika za razvoj kardiovaskularnih komplikacija, može se značajno popraviti kardiovaskularni ishod bolesnika koji se leče hemodijalizom.

Ključne reči: *faktori kardiovaskularnog rizika, mikroiflamacija, malnutricija, hemodijaliza*

INTRODUCTION

Cardiovascular diseases are a leading cause of mortality in patients treated with haemodialysis (1, 2). Traditional and non-traditional risk factors for cardiovascular complications in haemodialysis patients are numerous. Traditional risk factors include high blood pressure, lipid metabolism disorders, diabetes mellitus, obesity, cigarette smoking and reduced physical activity. Non-traditional risk factors can be metabolic (microinflammation, hyperhomocysteinaemia, high concentration of asymmetric dimethylarginine, oxidative stress, malnutrition, secondary hyperparathyroidism) or haemodynamic (anaemia, sodium and water retention and high blood flow through the vascular access for haemodialysis) (table 1) (3-5). Timely detection of risk factors and adequate therapy can significantly reduce cardiovascular morbidity and mortality in patients treated with haemodialysis (3-5).

Table 1: Risk factors for development of cardiovascular complications in dialysis patients

CARDIOVASCULAR RISK FACTORS		
TRADICIONAL	**NONTRADICIONAL**	
Hypertension	**METABOLIC**	**HEMODYNAMIC**
Hyperlipidaemia	Microinflammation	Anaemia
Diabetes mellitus	Hyperhomocisteinaemia	Na^+ and H_2O retention
Cigarette smoking	Oxidative stress	Q_{AV} > 1000 ml/min
Obesity	Malnutrition/hypoalbuminaemia	
Reduced physical activity	Secondary hyperparathyroidism	

Q_{AV} - flow through vascular haemodialysis access

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METABOLIC RISK FACTORS

Microinflammation

Microinflammation is a risk factor for atherosclerotic cardiovascular complications in patients treated with haemodialysis (6, 7). The normal concentration of C-reactive protein (CRP) in plasma is ≤ 5 mg/L, and concentrations of CRP > 10 mg/L indicate the presence of microinflammation and an elevated risk for the development of cardiovascular complications (6, 7). Microinflammation is present in 30-50% of patients. The quality of water for dialysis, biocompatibility of the dialysis membrane and vascular access for haemodialysis are all key factors in the provocation and maintenance of low degree chronic microinflammation in these patients (6-8). Microinflammation plays an important role in the process of atherosclerosis, plaque formation and rupture (6-8). Haemodialysis patients with CRP concentration > 15.8 mg/L have a 2.4-fold greater risk for cardiovascular mortality in comparison to patients with CRP < 3.3 mg/L (9). Patients with plasma CRP concentration > 11.5 mg/L have a highly statistically significant decrease in survival in comparison with patients treated with haemodialysis with a CRP concentration < 2.6 mg/L (10). Notably, bicarbonate haemodialysis with polisulfonic biocompatible membrane along with the use of ultrapure haemodialysis solution contributes to a decrease in CRP levels (11).

Hyperhomocysteinaemia

Hyperhomocysteinaemia is a risk factor for atherosclerosis and cardiovascular complications in haemodialysis patients (12-14). It is defined as a plasma homocystein concentration ≥15 μmol/L and is present in over 80% of patients treated with haemodialysis (12-14). It is as a consequence of the decreased activity of key enzymes involved in homocystein metabolism (methionine synthase, N5,N10-methyl tetrahydrofolate reductase, cystation β-synthase, betain-homocystein methyltransferase) (12-14). Hyperhomocysteinaemia blocks the degradation of asymmetric dimethylarginine (ADMA), contributes to the accumulation of ADMA in blood vessel endothelial cells and triggers atherosclerosis (scheme 1) (12-14). The concentration of whole serum homocystein - tHcy is an independent predictor of cardiovascular mortality in patients treated with regular haemodialysis. Patients treated with haemodialysis who have plasma homocystein concentrations ≥ 37.8 μmol/L exhibit an 8.2-fold greater risk for cardiovascular mortality in comparison with patients who have serum homocystein levels ≤ 22.9 μmol/L (15). Use of folan, vitamin B6, vitamin B12 and active metabolites of folic acid significantly contributes to decreased serum homocystein concentrations in patients treated with haemodialysis (16).

High concentration of asymmetric dimethylarginine

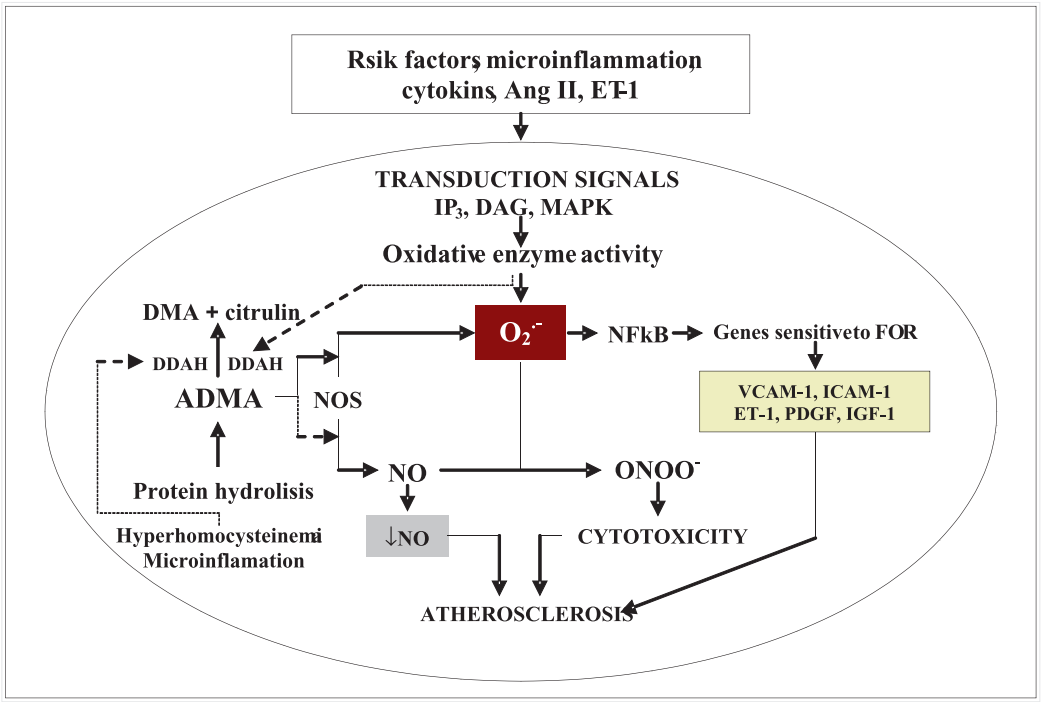
A high concentration of asymmetric dimethylarginine (ADMA) is a risk factor for cardiovascular complications in haemodialysis patients. It is defined as an ADMA concentration > 2.2 μmol/L and is due to decreased activity of the enzyme dimethylarginine dimethylhydrolase (DDHA) (17, 18). Microinflammation, diabetes mellitus, hyperhomocysteinaemia and oxidative stress significantly decrease the activity of this enzyme and increase the concentration of ADMA. ADMA blocks the production of nitrous oxide (NO) in endothelial cells and contributes to the development of atherosclerosis (scheme 1) (17, 18). Asymmetric dimethylarginine is an independent risk factor for left ventricle hypertrophy and a predictor of poor outcome in patients treated with haemodialysis (19). Increases in serum ADMA are accompanied by a 26% increase in overall mortality risk per μmol increase (20). Control of blood pressure, glycaemia, L-arginine and antioxidants improves the activity of DDAH and decreases the serum ADMA concentration (17-20).

Oxidative stress

Augmentation of oxidative stress is a risk factor for development of atherosclerotic cardiovascular complications in patients on haemodialysis (21). Oxidative stress and elevated oxLDL concentration block DDAH activity and decrease ADMA degradation. Accumulation of ADMA perturbs the function of the L-arginine/NO system in endothelial cells, resulting in decreased NO elaboration and the development of atherosclerosis (scheme 1) (21). Use of L-arginine, vitamin E and N-acetylcystein significantly contributes to decreasing oxidative stress and decreased risk of cardiovascular complications in patients treated with haemodialysis (22).

Malnutrition

In patients treated with haemodialysis, malnutrition due to insufficient protein consumption shows that there is microinflammation (23). Syndrome of malnutrition-inflammation (MICS) occurs due to appetite loss, insufficient nutrition, increased nutrient loss during haemodialysis, presence of uremic toxins, hypercatabolism produced by co-morbidities (diabetes mellitus, infection, sepsis, congestive heart failure), increased oxidative stress, and use of biocompatible dialysis membranes and conventional haemodialysis solution (23). As a consequence, inadequate response to erythropoietin, accelerated atherosclerosis and greater cardiovascular morbidity and mortality occur in patients treated with haemodialysis (23). Daily energy intake of 45 kcal/kgbm/day and protein intake of 1.5 g/kgbm/day lead to body mass growth and an increase in the concentration of serum albumin in patients treated with haemodialysis (23). Intradialysis parenteral nutrition (IDPN) along with appetite-stimulating medicines (megestrol acetate, L-carnitin) significantly improves nutritional status and outcomes in haemodialysis patients (23). Optimisation of dialysis treatment (with biocompatible dialysis membrane, dialysators with vitamin E, and an ultrapure solution for haemodialysis) also contributes to better outcomes in haemodialysis patients (23).



Scheme 1. Pathophysiological mechanisms of atherosclerosis development in dialysis patients

Ang II - angiotenzin II, ET-1 - endothelin 1, ADMA - asymmetric dimethylarginine, FOR - free oxygen radicals, NO - nitrous oxide, DDAH - dimethylarginine dimethylhydrolase, NFkB - nuclear factor, PDGF - platelate growth factor, NOS - nitrous oxide synthase, DMA - dimethylamin, IGF-1 - insulin like growth factor

Secondary hyperparathyroidism

Secondary hyperparathyroidism (SHTPH) frequently occurs in patients treated with regular haemodialysis. It is due to the decreased production of active metabolites of vitamin D3, hypocalcaemia and hyperphosphataemia, and its main clinical consequences are renal osteodystrophy with bone hypermetabolism, vascular and valvular calcifications and cardiovascular complications (24). Calcifications may occur in the tunica intima (atherosclerotic plaques), tunica media of the coronary vessels or in heart valves (24). Calcifications in the intima of the coronary arteries cause shrinkage of their lumen and result in the inability to supply the myocardium sufficiently (ischaemia). In addition, plaque rupture can cause acute coronary syndrome. Calcifications in media make arteries harder, increase the afterload of the left ventricle and contribute to its hypertrophy. Heart valve calcifications lead to aortic and mitral valve stenosis (24). Treatment of secondary hyperparathyroidism should enable patients to reach target endpoints for parameters involved in the metabolism of calcium and phosphate [intact parathormone (iPTH) 150 - 300 pg/mL (16.5 - 33.0 mg/dL) serum calcium concentration (Ca2+) 2.10 - 2.37 mmol/L (8.4 - 9.5 mg/dL), serum phosphate concentration (PO43-) 1.13 - 1.78 mmol/L (3.5 - 5.5 mg/dL), solubility product (CaxPO4) < 4.5 mmol2/L2 (< 55 mg2/dL2)] (25). In patients treated with regular haemodialysis, monitoring of calcium and phosphate should take place once every month, and monitoring of parathormone should be done every three months (25). Phosphate intake restriction (10 mg/kgbm/day), phosphate binders without calcium, new vitamin D metabolites and calcimimetics contribute to better control of secondary hyperparathyroidism and decrease the risk of cardiovascular morbidity and mortality in patients treated with haemodialysis (scheme 2) (26-30).

HAEMODYNAMIC RISK FACTORS

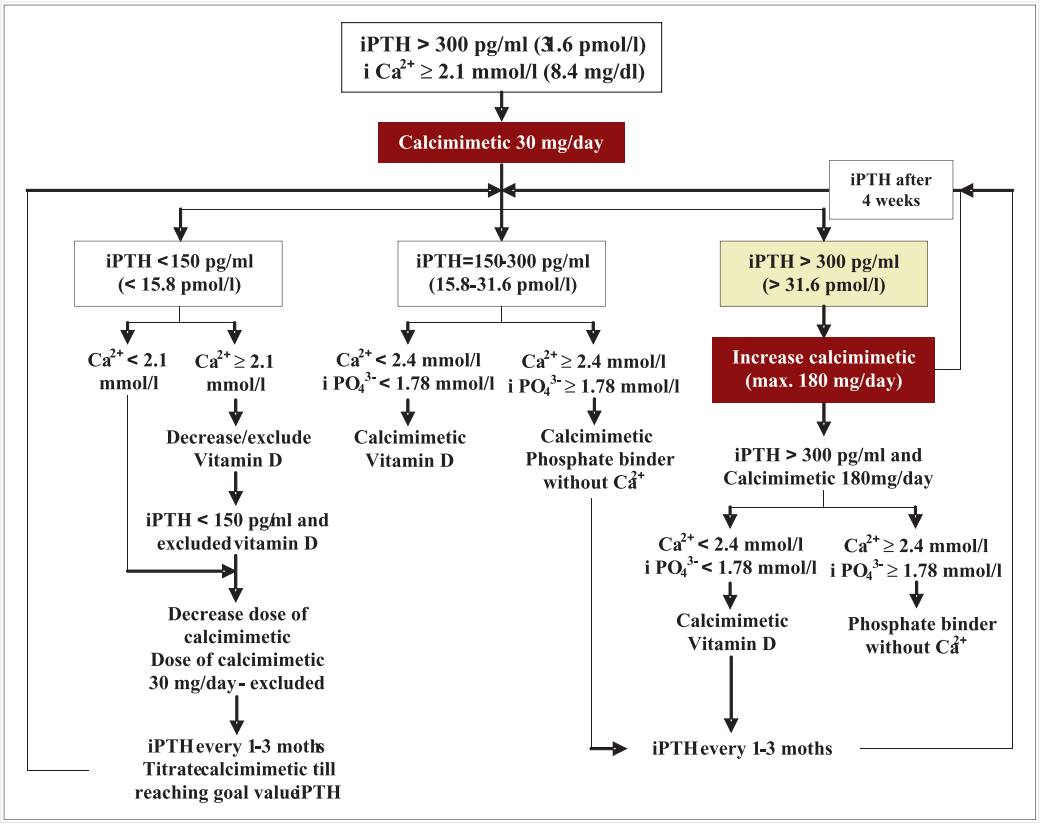
Anaemia

Anaemia is an independent risk factor for left ventricle hypertrophy in patients treated with haemodialysis (31). It is defined as a concentration of haemoglobin < 110 g/L and is present in over 90% of patients on haemodialysis (31, 32). Anaemia mostly comes from insufficient endogenous erythropoietin, and its main drawbacks to the cardiovascular system are decreased blood viscosity, low peripheral resistance (vasodilatation due to hypoxia), tachycardia and increased cardiac output (scheme 3) (31-35). By activation of haemodynamic adaptation mechanisms, anaemia overloads the left ventricle with excessive volume and causes its hypertrophy (31-35). Administration of erythropoietin enables patients to reach endpoint targets for haematocrit and haemoglobin (haemoglobin 110-120 g/L) and reduces left ventricle hypertrophy (36, 37).

Na+ and H2O retention

Interdialysis weight gain (IDWG) is a direct consequence of increased mineral and water intake in the interdialysis interval. IDWG is calculated from the following formula: IDWG% = [IDWG (kg)/DW (kg)] x 100%, where IDWG is interdialysis weight gain and DW is a patient's dry weight (38). Patients with an IDWG < 3.0% have a statistically lower body mass index in comparison to patients with IDWG > 3.0% (38). Increased mineral and water intake (IDWG > 5.0%) leads to left ventricle volume over-

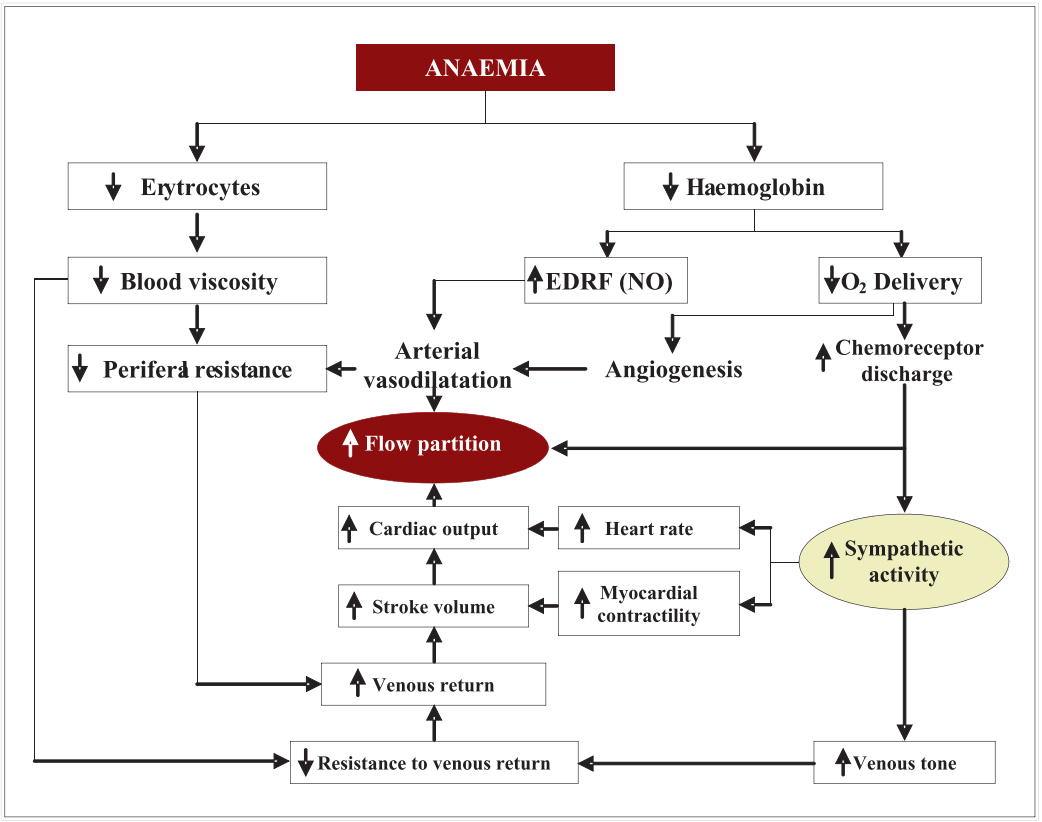
Scheme 2. Protocol for secondary hyperparathyroidism treatment in dialysis patients



Modified according to reference [28].

Conversion units: calcium 2,1 mmol/l = 8,4 mg/dl, calcium 2,4 mmol/l = 9,5 mg/dl, phosphate: 1,8 mmol/l = 5,5 mg/dl, iPTH 300 pg/ml = 31,8 pmol/l, iPTH 150 pg/ml = 15,9 pmol/l, iPTH 100 pg/ml = 10,6 pmol/l, iPTH 800 pg/ml = 84,8 pmol/l.

Scheme 3. Influence of anaemia on remodelling the cardiovascular system in dialysis patients



Modified according to reference [35].

EDRF - endotel relaxing factor

load, increased arterial pressure and left ventricle hypertophy (38). Restriction of mineral intake (2.0 g/24h NaCl) as well as liquids in between two dialysis sessions decreases the risk of development of cardiovascular complications in dialysis patients (38).

Increased flow through the vascular haemodialysis access

Increased flow through the vascular haemodialysis access is a risk factor for development of cardiovascular complications (table 2) (39, 40). Normal flow through the arteriovenous fistula (AVF) is 100350 cm/s, and normal blood flow is 500 - 1000 mL/min (39, 40). Increased flow through the vascular haemodialysis access is associated with increased end diastolic diameter (EDD) and increased end diastolic left ventricle volume (EDV) (41). Blood flow through the vascular haemodialysis access (QAV > 1000 mL/min) overloads the left ventricle with excess volume and stimulates a series of adaptive processes resulting in left ventricle remodelling (scheme 4) (42, 43).

Strategy for prevention of development of cardiovascular complications

	RISK AFCTORS
1.	Increased flow through the access - Q_{AV} > 1000 ml/min a) congestive heart failure b) distal steal phenomenon
2.	Decreased flow through the access - Q_{AV} < 300 ml/min a) inadequate haemodialysis - Kt/V index < 1,2 b) malnutrition - hypoalbuminaemia (albumin < 35 g/l)
3.	Infection of vascular access a) infectious endocarditis b) chronic microinflammation - CRP > 10 mg/l

Table 2: Risk factors for the development of cardiovascular complications in dialysis patients

Determination of the most sensitive parameters for detection of patients with a high risk of development of cardiovascular complications and early detection of cardiovascular risk factors enables timely and adequate therapy. This provides for a high survival rate and better quality of life of patients with end stage renal disease (44-49).

Cardiovascular risk factor modification can significantly improve cardiovascular outcomes in dialysis patients. Strict volume arterial pressure control, adequate dialysis dose, correction of anaemia by the use of erythropoietin, using carvedilol in patients with dilative myopathy, and secondary hyperparathyroidism therapy (calcimimetics in the therapy of secondary hyperparathyroidism) significantly improve cardiovascular outcomes in dialysis patients (table 3) (50, 51).

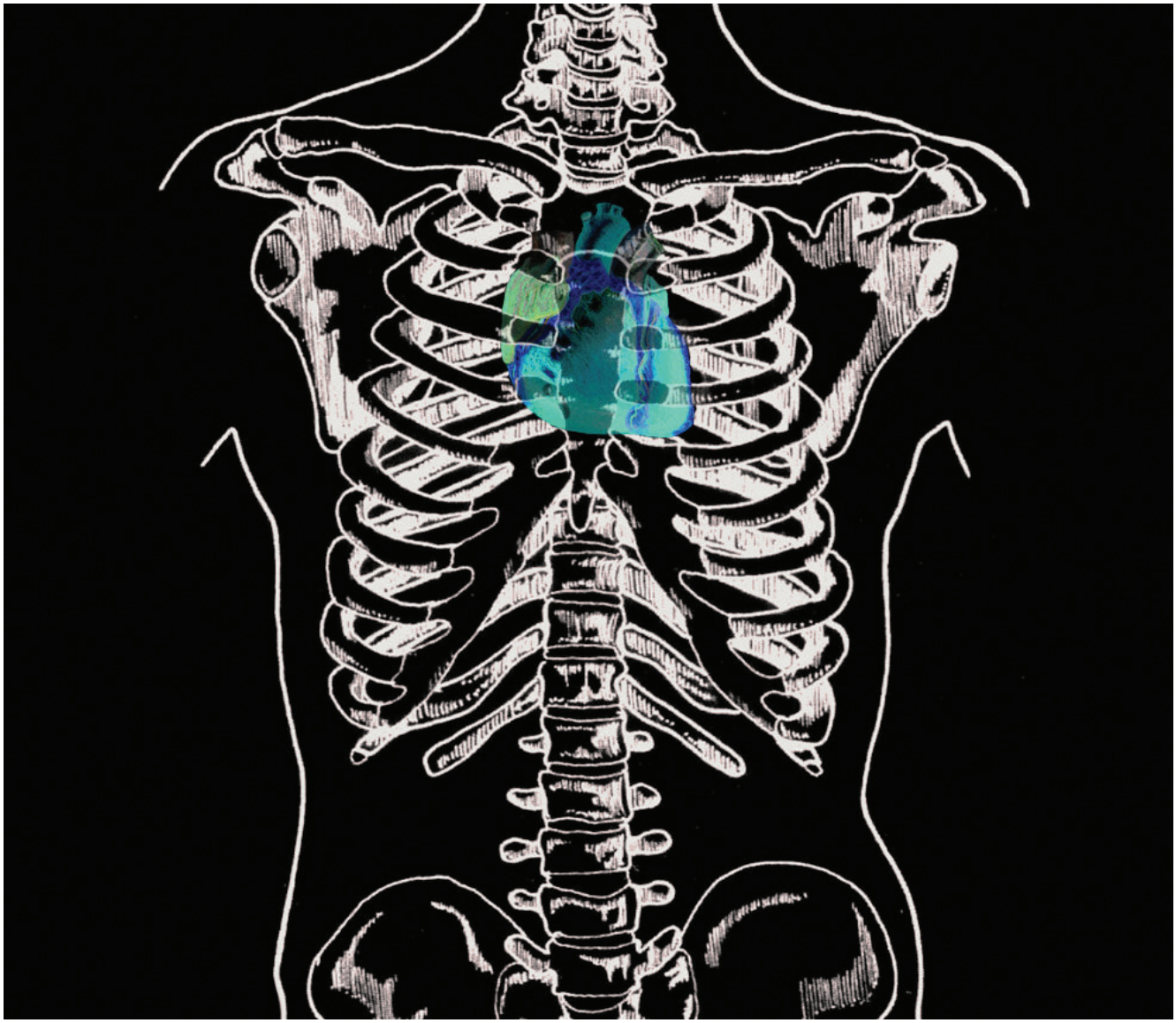
Overall cardiovascular risk in dialysis patients is the sum of traditional (TRF) and non-traditional risk factors (NTRF), uraemia-related risk factors (URRF) and dialysis technology-related risk factors (DTRRF) (52). Components of the dialysis procedure directly associated with increased risk of cardiovascular complications include the following: dialysator type (coefficient of ultrafiltration, dialysis membrane type, biocompatibility of dialysis membrane), microbiological quality of water and dialysis solutions, therapeutic modality and online monitoring (52, 53). Adequate dialysis procedure can greatly improve outcome in patients treated with regular haemodialysis (52, 53).

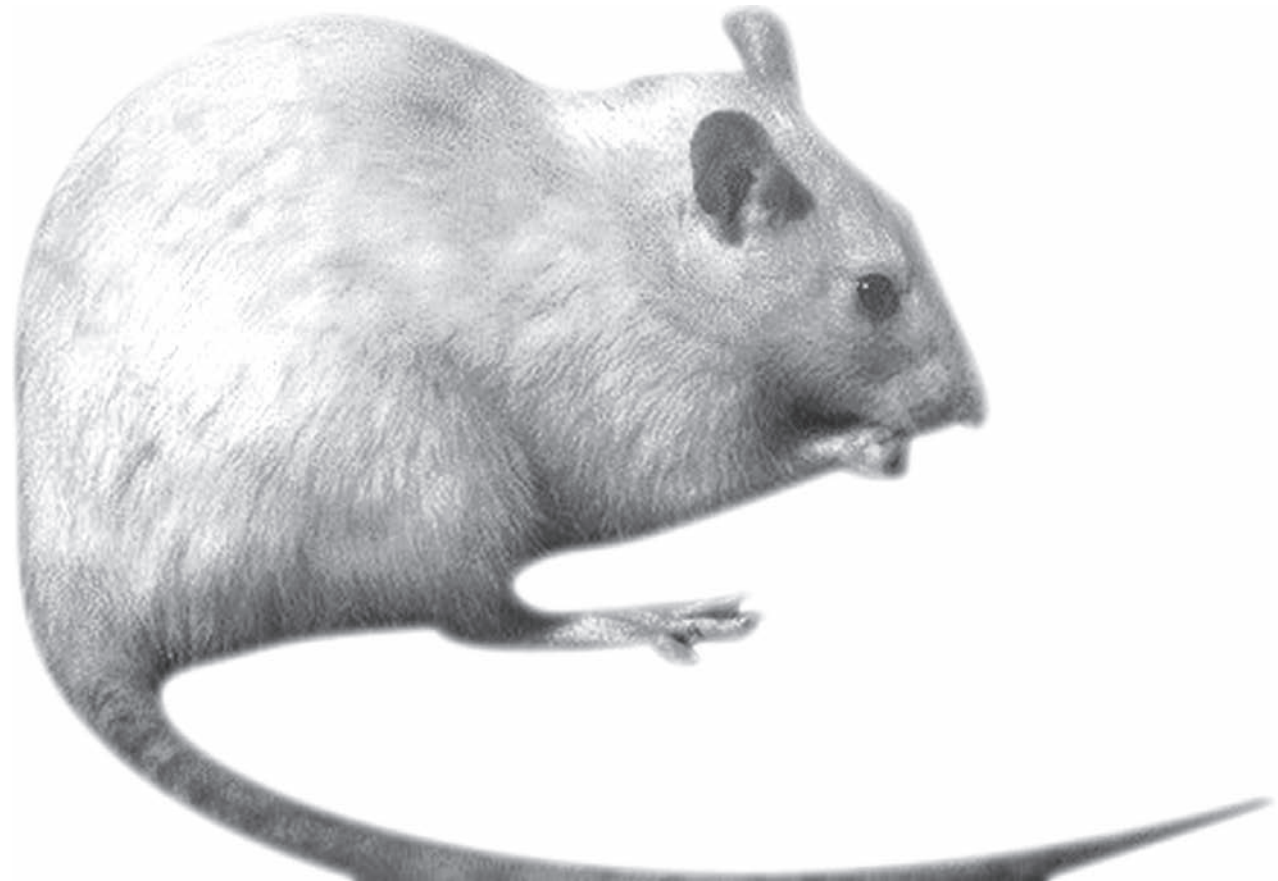
REFERENCES

1. Parfrey PS. Cardiac disease in dialysis patients: diagnosis, burden of disease, prognosis, risk factors and management. Nephrol Dial Transplant 2000; 15(Suppl 5): 5868.
2. Petrović D, Stojimirović B. Cardiovascular morbidity and mortality in hemodialysis patients - epidemiological analysis. Vojnosanit Pregl 2008; 65(12): 893-900. (in Serbian)
3. Rigatto C, Parfrey PS. Uraemic Cardiomyopathy: an Overload Cardiomyopathy. J Clin Basic Cardiol 2001; 4(2): 93-5.
4. Zoccali C, Mallamaci F, Tripepi G. Novel Cardiovascular Risk Factors in End-Stage Renal Disease. J Am Soc Nephrol 2004; 15(Suppl 1): 77-80.
5. Zoccali C. Tradicional and emerging cardiovascular and renal risk factors: An epidemiological perspective. Kidney Int 2006; 70(1): 26-33.
6. Lacson E, Levin NW. C-Reactive Protein and End-Stage Renal Disease. Semin Dial 2004; 17(6): 438-48.
7. Petrović D, Obrenović R, Poskurica M, Stojimirović B. Correlation of C-reactive protein with echocardiographic parameters of hypertrophic and ischemic heart disease in patients on regular hemodialyses. Med Pregl 2007; 50(Suppl 2): 160-4. (in Serbian)
8. Galle J, Seibold S, Wanner C. Inflammation in Uremic Patients:What Is the Link? Kidney Blood Press Res 2003; 26(2): 65-75.
9. Wanner C, Zimmermann J, Schwedler S, Metzger T. Inflammation and cardiovascular risk in dialysis patients. Kidney Int 2002; 61(Suppl 80): 99-102.
10. Yeun RA, Levine RA, Mantadilok V, Kaysen GA. C-reactive protein predicts all-cause cardiovascular mortality in hemodialysis patients. Am J Kidney Dis 2000; 35(3): 46976.
11. Ward RA. Ultrapure Dialysate. Semin Dial 2004; 17(6) : 489-97.
12. Friedman AN, Bostom AG, Selhub J, Levey AS, Rosenberg IH. The Kidney and Homocysteine Metabolism. J Am Soc Nephrol 2001; 12(12): 2181-9.
13. Petrović D, Stojimirović B. Homocisteine as risk factor for cardiovascular complications in hemodialysis patients. In: Radenković S, ed. Cardionephrology 2. Naiss: GIP "PUNTA", 2005: 31-6. (in Serbian)
14. Petrović D, Stojimirović B. Hyperhomocysteinemia - a risk factor for development of ischemic heart disease. In: Poskurica M, ed. Ischemic heart disease in patients with end-stage renal failure. Kragujevac: Inter Print, 2007: 71-8. (in Serbian)

15. Mallamaci F, Zoccali C, Tripepi G, Fermo I, Benedetto FA, Cataliotti A, et al. Hyperhomocysteinemia predicts cardiovascular outcomes in hemodialysis patients. Kidney Int 2002; 61(2): 609-14.
16. Buccinati G, Raselli S, Baragetti I, Bamonti F, Corghi E, Novembrino C, et al. 5-methyltetrahydrofolate restores endothelial function in uraemic patients on convective haemodialysis. Nephrol Dial Transplant 2002; 17(5): 85764.
17. Fliser D, Kielstein JT, Haller H, Bode-Böger SM. Asymmetric dimethylarginine: A cardiovascular risk factor in renal disease? Kidney Int 2003; 63(Suppl 84): 37-40.
18. Cooke JP. Asymmetrical Dimethylarginine: The Über Marker? Circulation 2004; 109(15): 1813-8.
19. Zoccali C, Mallamaci F, Maas R, Benedetto FA, Tripepi G, Malatino LS, et al. Left ventricular hypertrophy, cardiac remodeling and asymmetric dimethylarginine (ADMA) in hemodialysis patients. Kidney Int 2002; 62(1): 339-45.
20. Zoccali C, Bode-Böger SM, Mallamaci F, Benedetto FA, Tripepi G, Malatino LS, Cataliott A, et al. Plasma concentration of asymmetrical dimethylarginine and mortality in patients with end-stage renal disease: a prospective study. Lancet 2001; 358(9299): 2113-7.
21. Taki K, Takayama F, Tsuruta Y, Niwa T. Oxidative stress, advanced glycation end product, and coronary artery calcification in hemodialysis patients. Kidney Int 2006; 70(1): 218-24.
22. Johnson DW, Craven AM, Isbel NM. Modification of cardiovascular risk in hemodialysis patients: An evidence-based review. Haemodialysis Int 2007; 11(1): 1-14.
23. Kalantar-Zadeh K, Ikizler TA, Avram MM, Kopple JD. Malnutrition-inflammation complex syndrome in dialysis patients: Causes and consequences. Am J Kidney Dis 2003; 42(5): 864-8.
24. Cannata-Andia JB, Carrera F. The Pathophysiology of Secondary Hyperparathyroidism and the Consequences of Uncontrolled Mineral Metabolism in Chronic Kidney Disease: The Role of COSMOS. Nephrol Dial Transplant 2008; 1(Suppl 1): 29-35.
25. National Kidney Foundation. Clinical Practice Guidelines for Bone Metabolism and Disease in Chronic Kidney Disease. Am J Kidney Dis 2003; 42(4 Suppl 3): 1-201.
26. Chertow GM, Blumenthal S, Turner S, Roppolo M, Stern L, Chi EM, et al. Cinacalcet Hydrochloride (Sensipar) in Hemodialysis Patients on Active Vitamin D Derivatives with Controlled PTH and Elevated Calcium x Phosphate. Clin J Am Soc Nephrol 2006; 1(2): 305-12.
27. Chertow GM, Pupim LB, Block GA, Correa-Rotter R, Drueke TB, Floege J, et al. Evaluation of Cinacalcet Therapy to Lower Cardiovascular Events (EVOLVE): Rationale and Design Overview. Clin J Am Soc Nephrol 2007; 2(5): 898-905.
28. Messa P, Macario F, Yaqoob M, Bouman K, Braun J, von Albertini B, et al. The OPTIMA Study: Assessing a New Cinacalcet (Sensipar/Mimpara) Treatment Algorithm for Secondary Hyperparathyroidism. Clin J Am Soc Nephrol 2008; 3(1): 36-45.
29. Bushinsky DA, Messa P. Efficacy of Early Treatment with Calcimimetics in Combination with Reduced Doses of Vitamin D Sterols in Dialysis Patients. NDT plus 2008; 1(Suppl 1): 18-23.
30. Petrović D, Stojimirović B. Secondary hyperparathyroidism - a risk factor for development of cardiovascular complications in hemodialysis patients. Med Pregl 2009; (in press).
31. Stojimirović B, Petrović D, Obrenović R. Left ventricular hypertrophy in hemodialysis patients: importance of anaemia. Med Pregl 2007; 50(Suppl 2): 155-9. (in Serbian)
32. Foley RN, Parfrey PS. Anemia as a Risk Factor for Cardiac Disease in Dialysis Patients. Semin Dial 1999; 12(2): 84-6.
33. Levin A. Anaemia and left ventricular hypertrophy in chronic kidney disease populations: A review of the current state of knowledge. Kidney Int 2002; 61(Suppl 80): 35-8.
34. Petrović D, Stojimirović B. Left ventricular hypertrophy in patients treated with regular hemodialyses. Med Pregl 2008; 61(7-8): 369-74. (in Serbian)
35. London GM. Left ventricular hypertrophy: why does it happen? Nephrol Dial Transplant 2003; 18(Suppl 8): 2-6.
36. National Kidney Foundation. Clinical Practice Guidelines for Anemia of Chronic Kidney Disease: Update 2000. Am J Kidney Dis 2001; 37(Suppl 1): 182-238.
37. Massy ZA, Kasiske BL. Prevention of cardiovascular complications in chronic renal disease. In: Cardiovascular Disease in End-stage Renal Failure. Loscalzo J, London GM, editors. New York: The Oxford University Press, 2000. p. 463-81.
38. Lopez-Gomez JM, Villaverde M, Jofre R, et al. Interdialytic weight gain as a marker of blood pressure, nutrition, and survival in hemodialysis patients. Kidney Int 2005; 67(Suppl 93): 63-8.
39. Allon M. Current Management of Vascular Access. Clin J Am Soc Nephrol 2007; 2(4): 786-800.
40. Jagić N, Petrović D, Miloradović V, Novaković B. Clinical importance of early detection of vascular access failure in haemodialysis patients. Medicus 2006; 7(3): 103-6. (in Serbian)
41. Petrović D, Stojimirović B. Vascular access blood flow for hemodialysis - a risk factor for development of cardiovascular complications in hemodialysis patients. Med Pregl 2007; 60(3-4): 183-6. (in Serbian)
42. Dikow R, Schwenger V, Zeier M, Ritz E. Do AV Fistuals Contribute to Cardiac Mortality in Hemodialysis Patients? Semin Dial 2002; 15(1): 14-7.
43. MacRae JM, Levin A, Belenkie I. The Cardiovascular Effects of Arteriovenous Fistulas in Chronic Kidney Disease: A Cause for Concern? Semin Dial 2006; 19(5): 349-52.
44. Petrović D, Jagić N, Miloradović V, Stojimirović B. Clinical importance of biochemical markers of cardiac damage in hemodialysis patients. Ser J Exp Clin Res 2008; 9(1): 5-8. (in Serbian)

45. Zoccali C, Tripepi G, Mallamaci F. Predictors of Cardiovascular Death in ESRD. Semin Nephrol 2005; 25(6): 358-62.
46. Apple FS, Murakami MAM, Pearce LA, Herzog CA. Multi-Biomarker Risk Stratification of N-Terminal Pro-B-Type Natriuretic Peptide, High-Sensitivity C-Reactive Protein, and Cardiac Troponin T and I in End-Stage Renal Disease for All-Cause Death. Clin Chem 2004; 50(12): 2279-85.
47. Mallamaci F, Tripepi G, Cutrupi S, Malatino LS, Zoccali C. Prognostic value of combined use of biomarkers of inflammation, endothelial dysfunction, and myocardiopathy in patients with ESRD. Kidney Int 2005; 67(6): 2330-37.
48. Petrovic D, Obrenovic R, Stojimirovic B. Cardiac troponins and left ventricular hypertrophy in hemodialysis patients. Clin Lab 2008; 54(5-6): 145-52.
49. McIntyre CW, John SG, Jafferies HJ. Advances in the cardiovascular assessment of patients with chronic kidney disease. NDT Plus 2008; 1(6): 383-9.
50. Bossola M, Tazza L, Vulpio C, Luciani G. Is Regression of Left Ventricular Hypertrophy in Maintenance Hemodialysis Patients Possible? Semin Dial 2008; 21(5): 422-30.
51. Seibert E, Kuhlmann MK, Levin NW. Modifiable Risk Factors for Cardiovascular Disease in CKD Patients. In: Cardiovascular Disorders in Hemodialysis. Ronco C, Brendolan A, Levin NW (eds). Contrib Nephrol, Basel, Karger, 2005; 149: 219-29.
52. Bowry SK, Kuchinke-Kiehn U, Ronco C. The Cardiovascular Burden of the Dialysis Patient: The Impact of Dialysis Technology. In: Cardiovascular Disorders in Hemodialysis. Ronco C, Brendolan A, Levin NW (eds). Contrib Nephrol, Basel, Karger, 2005; 149: 230-9.
53. Ronco C, Bowry S, Tetta C. Dialysis Patients and Cardiovascular Problems: Can Technology Help Solve the Complex Equation? Blood Purif 2006; 24(1): 39-45.





THE EFFECTS OF DIFFERENT DOSES OF VARDENAFIL ON CORONARY AUTO-REGULATION IN ISOLATED RAT HEART

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EFEKAT RAZLIČITIH DOZA VARDENAFILA NA KORONARNU AUTOREGULACIJU IZOLOVANOG SRCA PACOVA

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ABSTRACT

Phospodiesterase-5 (PDE5) catalyses cyclic GMP (cGMP) degradation and regulates its intracellular content. Specific inhibition of that isoenzyme induces an increase in cGMP concentration, leading to smooth muscle cell relaxation and consequent vasodilatation. With regard to this information, the aim of our study was to compare the effects of different PDE5 inhibitors on coronary blood flow and the L-arginine/ NO system in isolated rat hearts. Hearts were isolated from male Wistar albino rats (n=12 rats) and perfused with buffer at a constant pressure. Coronary auto-regulation (CA) was investigated with follow-up of coronary perfusion pressure (CPP) changes from 40 to 120 cm H2O. After the first sequence of CPP changes (basic protocol), the hearts were perfused with vardenafil in different doses (10, 20, 50, 200 nM) alone or in combination with nitric oxide synthase inhibitor (L-NAME, 30 μM). During control conditions the hearts exhibited CA between 50 and 90 cm H2O, with a basal coronary flow (at 60 cm H2O) of 6.63⊠0.30 ml/min. Vardenafil induced significant vasodilatation at a dose of 200 nM (about 20% at all CPP-values) but not at other applied doses. Additional application of L-NAME induced decreases in coronary flow (CF) in all treated groups. Nevertheless, those effects were significant only at the lowest and highest doses of vardenafil.

Our findings clearly show that all estimated PDE5 inhibitors affect coronary auto-regulation, meditated by the L-arginine/NO system.

Key words: *PDE5 inhibitors, coronary circulation, NO*

SAŽETAK

Fosfodiesteraza 5 katalizuje razgradnju cikličnog GMP utičući na njegovu intraćelijsku koncentraciju. Specifična inhibicija tog izoenzima indukuje povećanje koncentracije cikličnog GMP, dovodeći do relaksacije glatkih mišićnih ćelija i posledične vazodilatacije. U skladu sa tim, cilj naše studije je bio da uporedimo efekte različitih inhibitora PDE5 na koronarni protok i L-arginin/NO sistem izolovanog srca pacova. Srca, izolovana iz pacova Wistar albino soja (muškog pola, n=12) su perfudovana pri konstantnom pritisku, tehnikom po Langendorff-u. Koronarna autoregulacija (KA) je ispitivana promenama koronarnog perfuzionog pritiska od 40 do 120 cm H2O i odredjivanjem vrednosti koronarnog protoka (KP) pri datim pritiscima. Nakon kontrolne serije eksperimenata (Bazični protokol), srca su perfundovana Vardenafilom u različitim dozama (10, 20, 50, 200 nM), kao i istim dozama Vardenafila u kombinaciji sa inhibitorom NO sintaze (L-NAME, 30μ M). U kontrolnim KA se odvijala izmedju 50 i 90 cm H2O, pri bazalnim vrednostima protoka (60 cm H2O) od 6,63± 0,30 ml/min. Vardenafil indukuje signifikantnu vazodilataciju u dozi od 200 nM (oko 20% od ukupnih CPP vrednosti), ali ne i pri ostalim aplikovanim dozama. Dodatna administracija L-NAME-a indukuje sniženje koronarnog protoka u svim tretiranim grupama. Ipak, samo pri najmanjim i najvišim vrednostima Vardenafila ovi efekti su bili sugnifikantni.

Naša saznanja jasno pokazuju da svi ispitivani inhibitori PDE5 ostvaruju uticaj na koronarnu autoregulaciju posredstvom L-arginin/ NO sistema.

Ključne reči: *inhibitor PDE5 - koronarna cirkulacija - NO*

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INTRODUCTION

Cyclic nucleotides (Cyclic adenosine 3'5'-monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)) function as major intracellular secondary messengers to mediate biological responses initiated by diverse extracellular signals (1). Cyclic nucleotide phosphodiesterases (PDEs) catalyse cAMP and cGMP and are important determinants regulating the intracellular concentrations and biological actions of both of these secondary messengers. Twelve families of these enzymes have been described so far (2).

Endothelial cells express three PDE isoforms: PDE2, PDE4 and PDE5, the last of which is a cGMP-specific PDE. This isoform is of special interest for our investigation regarding involvement in the nitric oxide (NO)/cGMP signalling pathway (3). Specifically, modulation of PDE5 activity directly influences all NO-mediated effects via the NO/cGMP cascade. Highly selective PDE5 inhibitors significantly amplified NO-mediated vascular effects in experimental (2) and clinical trials (4, 5). The most interesting clinical topic in a last few years is usage of a specific PDE5 inhibitor, sildenafil (Viagra®), in treatment of erectile dysfunction (6). PDE5 inhibitors cause vasorelaxation in various vascular beds by increasing intracellular cGMP levels in vascular smooth muscle (7). Hence, their intake is commonly associated with a moderate reduction in systolic and diastolic blood pressure in humans (5). Sildenafil has additional effects that are external to the corpus cavernosum, particularly strong vasodilatory effects highly selectivity for pulmonary circulation. Treatment of pulmonary hypertension with sildenafil showed beneficial effects that were amplified by the addition of inhaled NO through interference with the L-arginine/NO/cGMP system at distinct targets, thereby potentiating their actions (6, 8).

Recent studies have indicated that PDE5 inhibitors such as sildenafil, vardenafil or tadalafil induce preconditioning-like effects in the heart and protect against ischemia/reperfusion injury (9,10), leading to accumulated cGMP. The cGMP/PKG pathway has been shown in many reports to be involved in the protective signalling of preconditioning. Direct PKG activation with a cGMP analogue has proven to be protective (11), while receptor-mediated preconditioning could be blocked with a GC inhibitor (12). Salloum et al. (2007) used in situ rabbit hearts to demonstrate that sildenafil and vardenafil limited myocardial infarction when administered at the time of reperfusion in a model of ischemia/reperfusion by a mechanism that involved MkATP. Additionally, in a similar model performed on isolated rat hearts, du Toit et al. (2005) showed that low concentrations of sildenafil (20-50 nM) improve reperfusion function while higher concentrations (200 nM) worsen it.

For this purpose, the aim of our study was to evaluate the effects of a novel PDE5 inhibitor, vardenafil, on coronary auto-regulation in the isolated rat heart as one kind of ischemia/reperfusion model, with possible impact on the endothelial L-arginine/NO system.

MATERIAL AND METHODS

Isolated rat heart preparation

The hearts (n=12) excised from Wistar albino rats, male sex, body mass of about 200 g (obtained from Military Technical Institute, Belgrade, Serbia and Montenegro) were perfused with a Langendorff apparatus (Hugo Sachs Elektronik-Harvard Aparatus GmbH, March-Hugstetten, Germany). After short-term ether narcosis, the animals were killed by cervical dislocation (Schedule 1 of the Animals/Scientific Procedures, Act 1986, UK), with heparin premedication as an anticoagulant. After urgent thoracotomy and rapid heart arrest by superfusion with ice-cold isotonic saline, the hearts were rapidly excised and isolated. The aortas were cannulated and retrograde perfused according to the technique for constant pressure conditions. The composition of the non-recirculating Krebs-Henseleit perfusate was as follows (mmol/l): NaCl 118, KCl 4.7, CaCl2 × 2H2O 2.5, MgSO4 × 7H2O 1.7, NaHCO3 25, KH2PO4 1.2, glucose 11, pyruvate 2, equilibrated with 95% O2 plus 5% CO2 and warmed to 37 °C (pH 7.4). All hearts were electrically paced (5 V, 320 bpm) by the electronic stimulator (Hugo Sachs Elektronik-Harvard Aparatus GmbH) and constant left ventricular drainage through the dissected mitral valve was performed.

Physiological assay

After the heart perfusion had been set up, a 30-minute period was allowed for stabilisation of the preparation. During the stabilisation period, all hearts were challenged by short-term occlusions (5-30 seconds) as well as by bolus injection of 5 mmol/l adenosine (60 µl at a flow rate of 10 ml/min) to elicit maximal coronary flow. The hearts were discarded (about 25%) if the flow did not increase by 100% over the control value (for both tests). After the equilibration period, coronary perfusion pressure was lowered to 50 and 40 cm H2O and then gradually increased to 70, 80, 90, 100, 110 and 120 cm H2O to establish coronary autoregulation. When the flow was considered to be stable at each value of perfusion pressure, samples of the coronary effluent were collected. Properly performed control experiments were included in the study (i.e., the groups of the hearts in which the coronary perfusion pressure/coronary flow relationship were studied twice in the absence of any drug). It was essential to confirm that the preparation used was stable and that the responses to the first and second run of changes in perfusion pressure did not differ substantially. In the control protocol, preparation stabilisation was performed at a basal coronary perfusion pressure of 60 cm H2O for 30 minutes.

Experimental protocols

In the experimental protocol hearts were perfused with or without vardenafil (a specific PDE5 inhibitor) at different doses: 10, 20, 50 and 200 nM. In a second experimental protocol hearts were perfused with vardenafil (10, 20, 50 and 200 nM) plus an inhibitor of nitric oxide synthesis, Nω-nitro-L-arginine monomethyl ester (L-NAME, 30 µM) (3).

Statistical analysis

Values are expressed as means ± standard error (SE). Statistical analysis was performed by multifactorial analysis of variance for repeated measurements between subject factors as well as the Bonferroni test. P values less than 0.05 were considered to be significant.

RESULTS

Control conditions

The results showed that isolated rat heart auto-regulated between 50 and 90 cm H2O, which was reported previously (13) and slightly different in comparison to the auto-regulatory range of the isolated guinea pig heart (14). Coronary flow in the auto-regulatory range varied from 4.97±0.22 ml/min/g wt at 50 cm H2O to 6.68±0.25 ml/min/g wt at 90 cm H2O (Fig. 1-4).

PDE5 inhibition and coronary flow vs. PDE5+NOS inhibition and coronary flow

Perfusion with different concentrations of vardenafil induced a non-significant increase in coronary flow from 40 to 120 cm H2O (Fig 1A-3A), except in the high-dose group (200 nM) (Fig 4A). Additional NOS inhibition with L-NAME reversed vardenafil-induced effects on coronary flow (Fig 1B-4B), with statistical significance seen only at the lowest (10 nM) and highest (200 nM) concentrations of Vardenafil.

DISCUSSION

The present study showed that administering vardenafil does not significantly influence coronary flow in an isolated rat heart, except at the high dose of 200 nM. At all lower concentrations (10, 20, 50 nM) vardenafil did not influence coronary circulation. That result is in accordance with previous studies (10), with no clear explanation. That effect could be dependent on the activity of GC and the cGMP-dependent kinase (PKG).

Vardenafil and other PDE-5 inhibitors are established drugs in the treatment of erectile dysfunction in men. PDE enzymes hydrolyse the phosphodiester bond of the cyclic nucleotides cAMP and cGMP, which serve as second messengers in various cellular functions. Therefore, PDE inhibition can elevate intracellular cAMP or cGMP concentration, depending on their substrate specificity. Type-5 PDEs predominantly metabolise cGMP and are localised in many

tissues, including canine and mouse ventricular myocytes (15, 16, 17). In the heart, increasing intracellular cGMP via the addition of a cell-permeable cGMP analogue leads to reduced infarct size after ischemia/reperfusion with either pre-treatment (11) or treatment at reperfusion (18). Therefore, it seemed only logical that indirect cGMP elevation via PDE-5 inhibition would show similar effects. Ockaili (2002) was the first to show that sildenafil administered before ischemia reduced myocardial infarct size in an in situ rabbit heart model (19). Later, similar results could be shown for other PDE-5 inhibitors, such as vardenafil and tadalafil, in various models (20). Du Toit (2005) confirmed these results regarding sildenafil at the lowest concentration but not at the highest concentration, which was the basic postulate for our study (21).

However, PDE-5 inhibitors have proved to be effective not only when given before ischemia as preconditioning agents, but also when applied at reperfusion as post-conditioning agents. Salloum (2007) reported a marked reduction in infarct size in rabbit hearts in situ when sildenafil or vardenafil was administered at reperfusion (22). In the present study, we confirmed these data for vardenafil in isolated rat hearts with approximately the same concentration of the drug used in the earlier study. In our hands, administration of 10, 20 and 50 nM vardenafil had no sig-

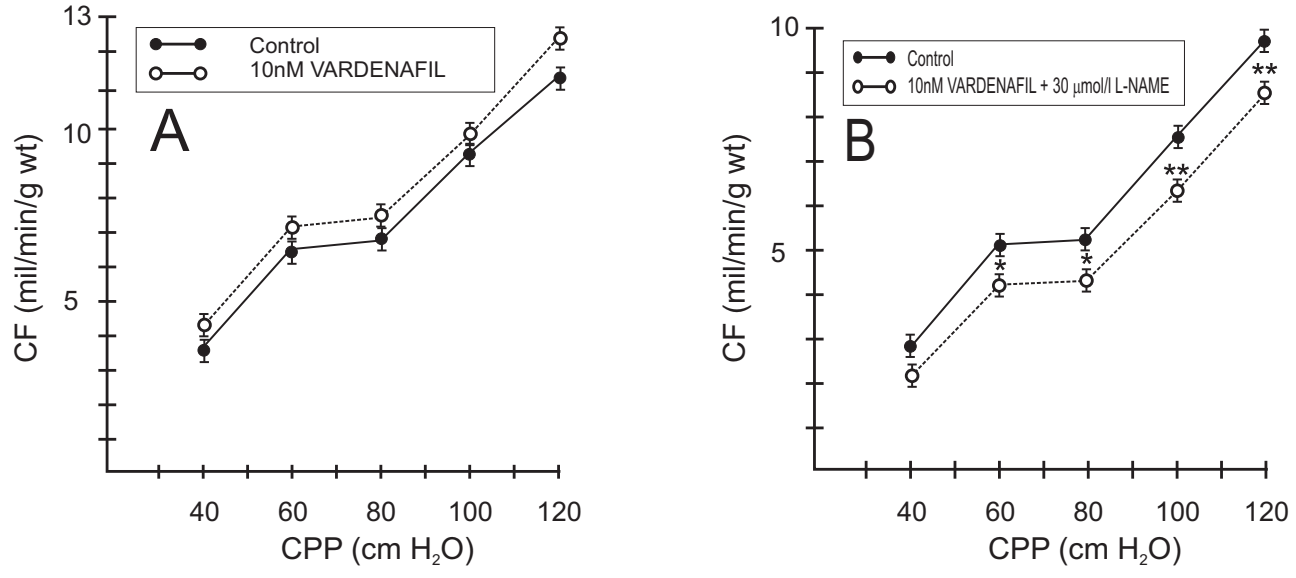

Fig. 1: Effects of A) 10 nM vardenafil or B) 10 nM vardenafil +30 µM L-NAME on coronary flow in isolated rat hearts compared to the control (n=6). The values are expressed as mean ± S.E.M., ** p< 0.01.

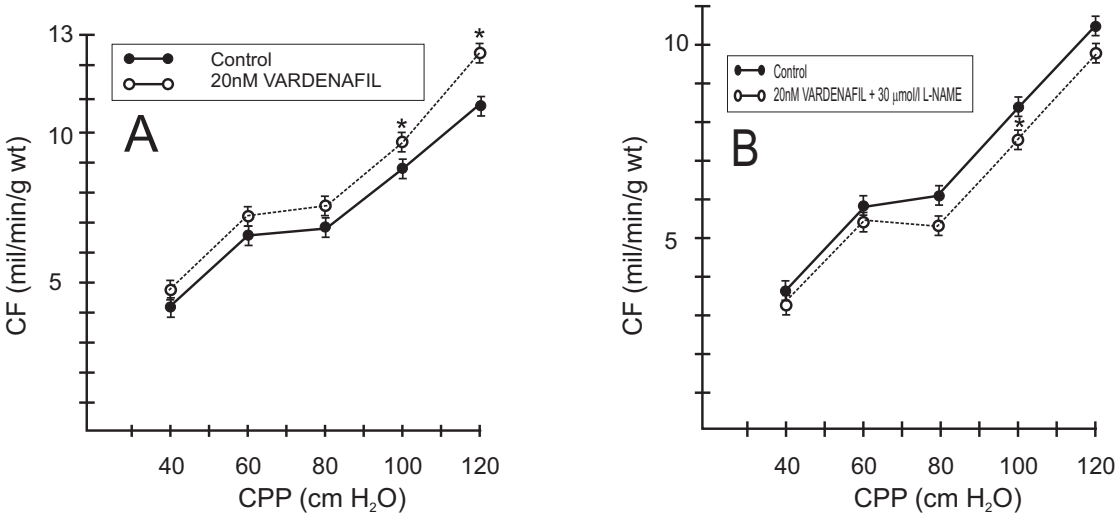

Fig. 2: Effects of A) 20 nM vardenafil or B) 20 nM vardenafil +30 µM L-NAME on coronary flow in isolated rat hearts compared to the control (n=6). The values are expressed as mean ± S.E.M., ** p< 0.01.

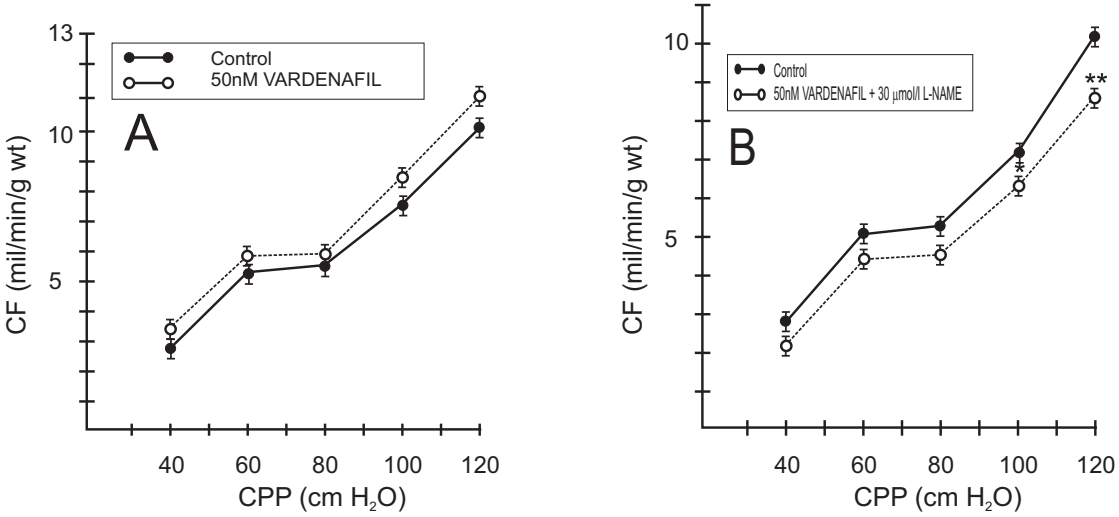

Fig. 3: Effects of A) 50 nM vardenafil or B) 50 nM vardenafil +30 µM L-NAME on coronary flow in isolated rat hearts compared to the control (n=6). The values are expressed as mean ± S.E.M., ** p< 0.01.

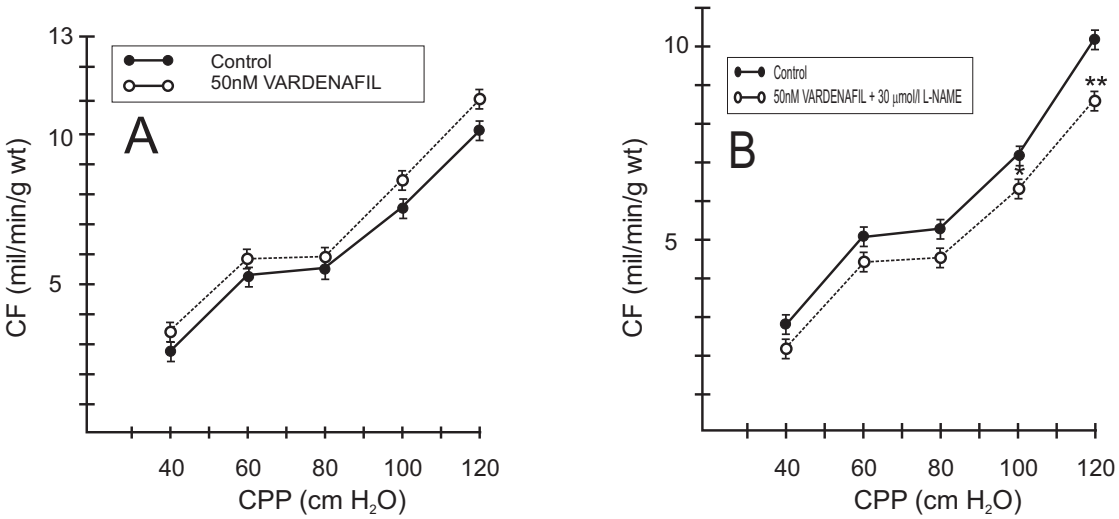

Fig. 4: Effects of A) 200 nM vardenafil or B) 200 nM vardenafil +30 µM L-NAME on coronary flow in isolated rat hearts compared to the control (n=6). The values are expressed as mean ± S.E.M., ** p< 0.01

nificant effect on the coronary vascular bed, while the high dose of 200 nM induced significant vasodilatation (Fig 1A-4A). This somewhat surprising result was in agreement with a report from du Toit et al. (2005), wherein they observed clear infarct size reduction with a pre-treatment of 50 nM sildenafil, but increasing the concentration to 200 nM removed this protective effect (21). Our results are also in accordance with a recent study on treated infarct size in isolated rat hearts (10).

Additional inhibition of the L-arginine/NO system reduced coronary flow in the presence of 10 and 200 nM vardenafil, but not when 20 and 50 nM were applied (Fig 1B-4B). That aspect of our results suggests that only protective and high doses of vardenafil might influence the coronary system via the NO system. Regarding to their combined intake with NO-liberating drugs such as organic nitrates (sublingual nitroglycerin, isosorbide dinitrate/mononitrate), this is absolutely contraindicated because such combinations may lead to excessive and uncontrolled hypotension (23) and these results shed new light on that problem. These deleterious interactions may be explained by the fact that nitrates (by leading to enhanced NO production and a rise in cGMP levels) and sildenafil (by inhibiting the decay of cGMP via PDE5) both interfere with the same system at distinct targets, thereby mutually potentiating their actions. Hence, the distinct interactional properties of NO-liberating drugs with PDE5 inhibitors may depend on the oxidative or anti-oxidative properties of the respective drug (7).

Our results suggest that the lowest (10 nM) and highest (200 nM) doses of vardenafil act via the NO system. Further measurement of oxidative stress parameters in that experimental model could indicate which dose of vardenafil is protective with regard to anti-oxidative potential.

REFERENCES

1. Jakovljević V: Efekti inhibitora različitih izoformi fosfodiesteraze (PDE) na koronarnu autoregulaciju. Doktorska disertacija, Medicinski fakultet, Kragujevac, 2004.
2. Beavo J. Cyclic nucleotide phosphodiesterase: functional implications and multiple isoforms. Phys Rev 1995; 75(4): 725-749
3. Kostić MM. A new bioregulatory system: nitric oxide from L-arginine. Iugoslav Physiol Pharmacol Acta 1993; 29(1): 3-34.
4. Halcox JPJ, Khaled RA, Zalos G et al. The effect of sildenafil on human vascular function,platelet activation and myocardial ischemia. JACC 2002; 40(7):1232-1240.
5. Jackson G, Benjamin N, Jackson N, Allen MJ. Effects of sildenafil citrate on human hemodynamics. Am J Cardiol 83 (5A): 13C-20C
6. Salonia A, Rigatti P, Montorsi F. Sildenafil in erectile dysfunction: a critical review. Curr Med Res Opin 2003; 19(4): 241-262.
7. Rosenkranz S, Brixius K, Halbach R, Diedrichs H, Schwinger HG R. Phosphodiesterase type 5 inhibitor sildenafil citrate does not potentiate the vasodilative properties of neivolol in rat aorta. Life sci 78 (2006) 1103-1107.
8. Zusman RM, Prisant LM, Brown Mj, 2000. Effect of sildenafil citrate on blood pressure and heart rate in man with erectile disfunctin talking concomitant antihypertensive medication. Sildenafil study group. J Hypertension 18 (12), 1865-1869
9. Kukrea R, Salloum F, Xi L, (2007). Anti-ischemic effects of sildenafil, vardenafil and tadalafil in heart. Int J Impot Res 19:226-227
10. Maas O, Donat U, Frenzel M, Rutz T, Kroemer HK, Felix SB, Krieg T. Vardenafil protects isolated rat hearts at reperfusion dependent on GC and PKG. Br J Pharmacol (2008) 154, 25-31
11. Qin Q, Yang XM, Cui L, Critz SD, Cohen MV, Browner NC at al. (2004). Exogenous NO triggers preconditioning via a cGMP and mitoKATP-dependent mechanism. Am J Physiol Heart Circ Physiol 287:H712-H718
12. Oldenburg O, Qin Q, Krieg T, Yang XM, Phillipp S, Critz SD at al. (2004). Bradycinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoKATP channel opening and leads to cardioprotection. Am J Physiol Heart Circ Physiol 286: H468-H476.
13. Leuhte HH, Schwaiblaimer M, Baumgartner RA, Neurohr CF, Kolbe T, Behr J. Hemodynamic response to sildenafil, nitric oxide, and iloprost in primary pulmonary hypertension. CHEST 2004; 125: 580-586.
14. Ziegler JW, Ivy DD, Wiggins JW, Kinsella JP, Clarke WR, Abman SH. Effects of dipyridamole and inhaled nitric oxide in pediatric patients with pulmonary hypertension. Am J Respir Crit Care Med 1998; 158: 1388-95.
15. Senzaki H, Smith CJ, Juang GJ, Isoda T, Mayer SP, Ohler A at al. Cardiac phosphodiesterase 5 (cGMP specific) modulates beta-adrenergic signaling in vivo and is down-regulated in heart failure. FASEB J 2001; 15:1718-26.
16. Bischoff E. Potency, selectivity and consequences of nonselectivity of PDE inhibition. Int J. Impot Res 16(Suppl 1) 2004; S11-S14.
17. Das A, Xi L, Kukreja RC. Phosphodiesterase 5 inhibitor sildenafil preconditions adult cardiac myocytes against necrosis and apoptosis. Essential role of nitric oxide signaling. J Biol Chem 2005; 280: 12944-55.
18. Yang XM, Philipp S, Downey JM, Cohen MV (2006). Atrial natriuretic peptide administered just prior to reperfusion limits infarction in rabbit hearts. Basic Res Cardiol 101: 311-318.
19. Ockali R, Salloum F, Hawkins J, Kukreja RC (2002). Sildenafil (Viagra) induces powerful cardioprotective effect via opening of mitochondrial K(ATP) channels in rabbits. Am J Physiol Heart Circ Physiol 283: H1263-H1269.
20. Ravipati G, McClung JA, Aronow WS, Peterson SJ, Frishman WH (2007). Type 5 phosphodiesterase in-

hibitors in the treatment of erectile dysfunction and cardiovascular disease. Cardiol Rev 15:76-86.
21. du Toit EF, Rossouw E, Salie R, Opie LH, Lochner A (2005). Effect of sildenafil on reperfusion function, infarct size, and cyclic nucleotide levels in the isolated rat heart model. Cardiovasc Drugs Ther 19: 23-21
22. Salloum F, Yin C, Xi L, Kukreja C. Sildenafil induces delayed preconditioning through inducible nitic oxide synthase-dependent pathway in the mouse heart. Circ Res 2003; 92:595-597.
23. Cheitlin, M.D., Hutter, A.M., Brindis Jr., R.G., Ganz, P., Kaul, S., Russell Jr., R.O., Zusman, R.M.,(1999). ACC/AHA expert consensus document. Use of sildenafil (Viagra) in patients with cardiovascular disease. American College of Cardiology/American Heart Association. J Am Coll Card 33 (1), 273-282.

INTRAMEDULLARY SPINAL CORD METASTASIS IN BREAST CARCINOMA

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INTRAMEDULARNA METASTAZA U KICMENOJ MOZDINI KOD KARCINOMA DOJKE

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ABSTRACT

Intramedullary spinal cord metastasis (ISCM) is a rare complication of some tumours. In this article, the authors present the case of a 55-year-old woman with breast carcinoma who presented with lumbar vertebral pain radiating to the lower limbs and weakness in the legs progressing to moderate paraparesis over a short interval of time. Eighteen months before onset of symptoms, the patient underwent partial mammaectomia with dissection axillae (level I-III) due to breast cancer. After clinical investigation, a total resection of the intramedullar tumour was made, and pathological findings showed metastatic carcinoma of the breast.

Key words: *spinal cord, intramedullary metastasis, breast carcinoma*

SAŽETAK

Intramedularne metastaze u kičmenoj moždini su rijetaka komplikacija.U radu je prikazan slučaj žene stare 55 godina sa kacinomom dojke kod koje se u kratkom vremenskom periodu pojavio bol u lumbalnoj regiji sa iradijacijom u donje ekstremitete praćen djelimičnom slabošću ekstremiteta srednje teškog stepena.Osamnest mjeseci prije pojave simptoma kod pacijentkinje je uradjena parcijalna lijeva mamektomija sa disekcijom pazušne jame(I-III) zbog kacinoma. Nakon kliničkog ispitivanja i uradjne nuklerne magnetne resonance, postavljena je dijagnoza. Intramedularni tumor je u cjelini odstranjen a patohistoloski nalaz je potvrdio metastaski tumor iz dojke.

Klučne rijeci: *kičmena moždina, intramedularna metastaza, karcinom dojke*

INTRODUCTION

Intramedullary spinal cord metastasis (ISCM) is rare, but with increasing use of magnetic resonance imaging (MRI), its occurrence is being encountered with increasing frequency. The outcome of surgical treatment is considered to be poor. The question of optimal treatment remains controversial.

CASE REPORT

A 55-year-old woman was admitted to the neurosurgery department with complaints of deep aching low back pain radiating in both legs with progressive weakness over one month, numbness in the lower extremities and sphincter disturbance. Eighteen months prior to the onset of symptoms, the patient underwent a partial left mammaectomia with dissection axillae (level I-III) due to breast cancer (Carcinoma ductale mammae invasivum HG3, NG3). Upon neurological investigation, there was muscle weakness and difficulty walking, bilateral positive Babinski's sings and increasing deep tendon reflexes, and sensory impairment appropriate to segmental level (conus medullaris).

MRI of the lumbar spine revealed an intramedullar mass lesion extending to the inferior two-thirds of L1, with hyperintensity on a T2-weighted image with contrast enhancement (Fig.1).

Laboratory findings were normal, and metastases were not found outside of the spinal cord (CT of brain and X-ray of lungs were normal).

The patient underwent laminectomy Th 12-L2 with total removal of the tumour by microsurgery (Fig.2)

The patient showed neurological improvement after surgical treatment. After eight months, the patient died of brain and lung metastases.

DISCUSSION

Primary spinal cord tumours represent about 15% of primary CNS tumours: extradural (45-55%), intradural but extramedullary (40-50%) and intramedullary (5%) [1,2].

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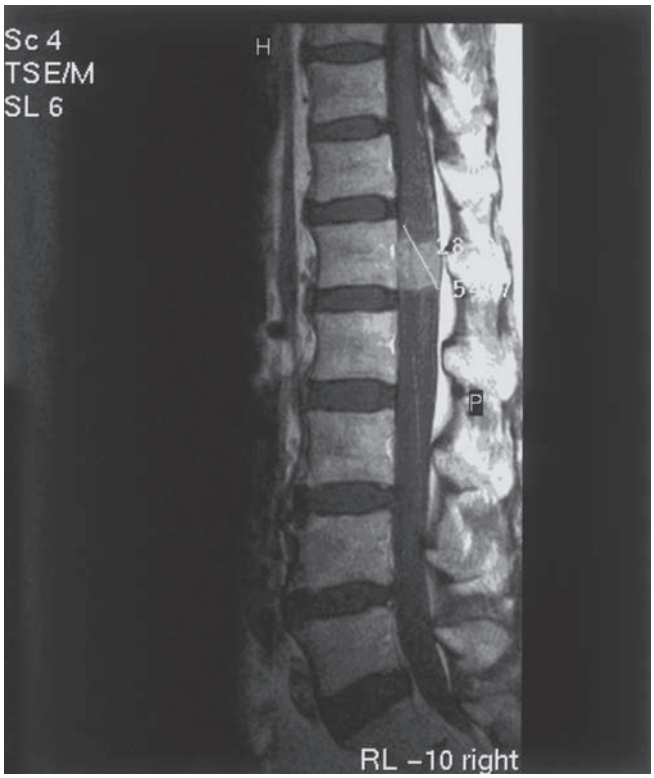

Fig. 1: MRI Intramedullary spinal cord metastasis (L1)

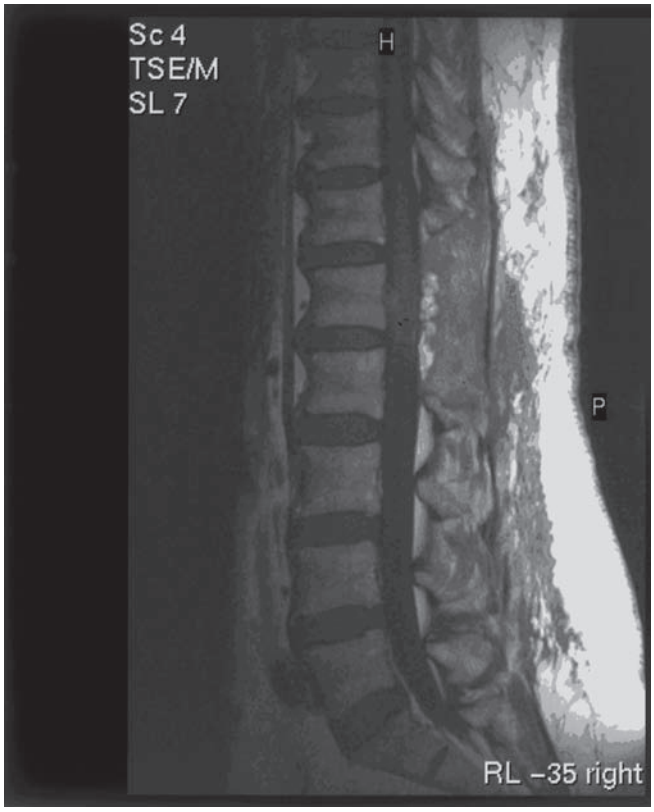

Fig. 2: MRI spinal cord (L1) after surgical intervention

Spinal epidural metastases occur in up to 10% of cancer patients at some time and present 94-96% of all spinal metastases, with the most common being spinal tumour [2]. Eighty-five percent of bony metastases in the vertebral column directly involve the spinal canal or the intervertebral foramina. Intradural but extramedullary metastases are rare (2-4%) and consist of leptomeningeal metastases of carcinoma or lymphoma, which cause malignant meningitis.

Intramedullary spinal cord metastases are very rare and account (1-3,4%) for symptomatic metastatic spinal cord lesions. Small cell lung carcinoma (49,1-64%), breast carcinoma (11-14,5%), melanoma (3,6-7,5%), colorectal (3-7,3%), lymphoma (3-12%), renal cell carcinoma (3-5,5%) and unidentified (1,8-3%) are the most commonly diagnosed primary tumours from which the metastasis arises [1,2,3,4,5]. Most lung tumours are thought to spread to the intramedullary spinal cord through the arterial route. The second way that tumours are thought to metastasise is via the vertebral venous plexus of Batson. Finally, the third method of spread to the spinal cord is by direct extension from the nerve roots or the cerebrospinal fluid with malignant cells from tumours found elsewhere in the central nervous system [5].

Clinical features of ISCM depend on the site and extent of the spinal cord lesion as well as the rate of growth. A central lesion initially damages second sensory neurons that cross to the lateral spinotalamic tract; pain and temperature sensations are impaired in the distribution of the involved segment. As the lesion expands, anterior horn cells are also involved, and lower motor neuron weakness occurs. Weakness and pain present early as compared to sensory loss. With a lesion in cervical region, the sensory deficit to pain and temperature extends downwards in a "cape"-like distribution. Involvement of the corticospinal tracts produces upper motor neuron symptoms in the limbs below the level of the lesion. The bladder is usually involved later. In the cervical cord, sympathetic involvement may produce unilateral or bilateral Horner's syndrome [6].

None of these features can reliably differentiate intramedullary spinal cord metastases from malignant extramedullary spinal cord compression; however, the duration of symptoms is generally shorter in the case of intramedullary spinal cord metastases.

The use of MRI has facilitated the identification and localisation of spinal cord tumours. MRI with contrast medium is now the method of choice to determine whether the tumour lies within or outside the dura or the spinal cord. The examination must involve both T1- and T2-weighted images, the former often repeated with gadolinium enhancement. MRI can differentiate a syrinx or a cystic swelling within spinal cord from the solid intramedullary tumour. Metastases appear as lumps that enhance within the cord. Myelography and CT myelography generally give negative findings, especially in patients with small lesions that do not alter the contour of the spinal cord [7]. Lumbar puncture may precipitate acute deterioration if there is cord compression and may damage the spinal cord if it is tethered to the lower lumbar or sacral vertebral bodies. CSF cytology may reveal malignant cells.

While surgery is increasingly recommended for benign and malignant primary spinal cord tumours, the role of surgery in spinal metastasis, i.e., cancer that has spread to

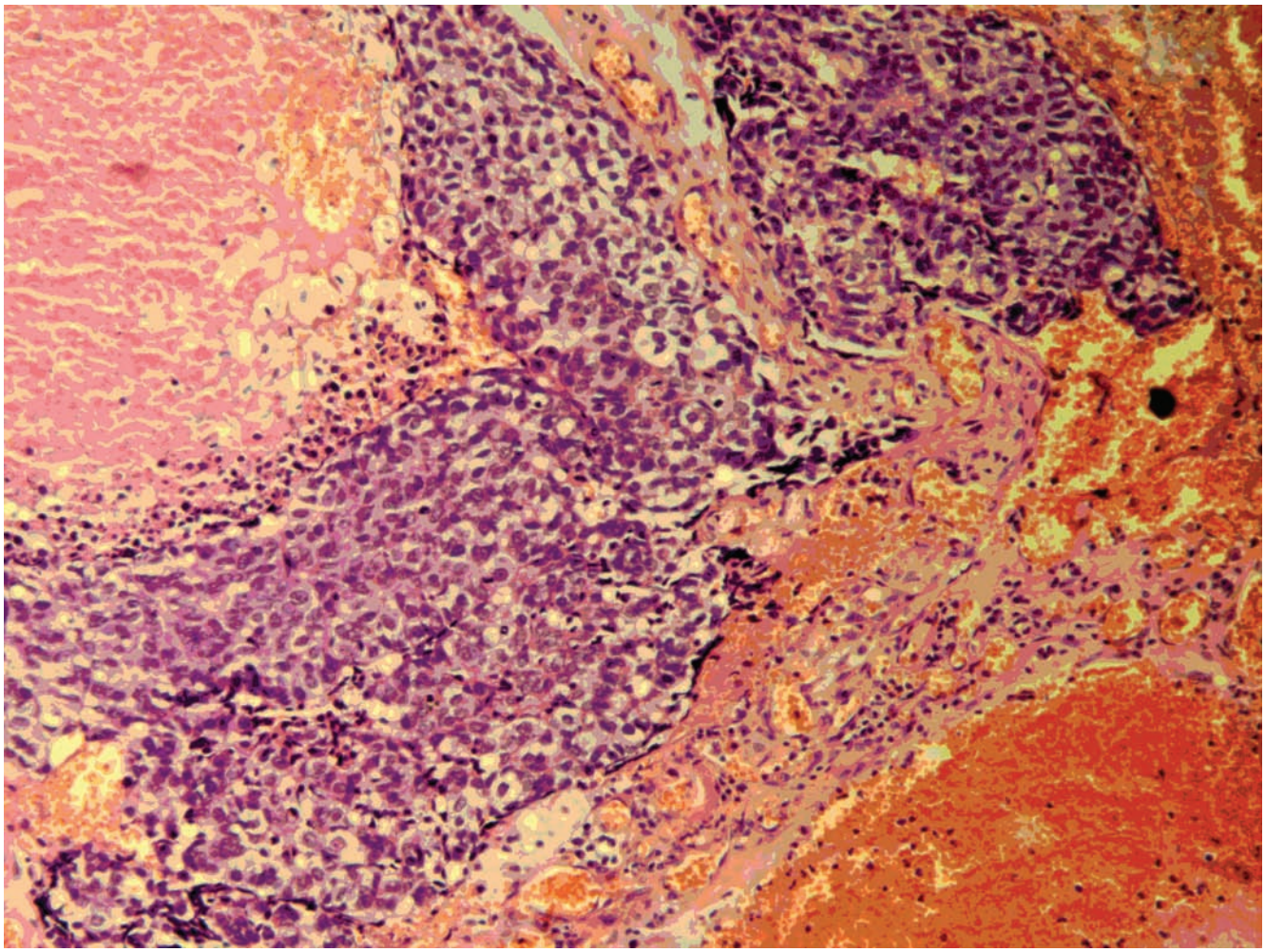

Fig. 3: Carcinoma ductale mammae metastaticum in medullae spinalis 100x

the spine, is controversial. Recent developments in imaging as well as new surgical tools and techniques such as the use of an ultrasonic aspirator and laser have significantly expanded the role of surgery as an intervention. Some doctors may only recommend surgery for patients with a single metastatic site and no evidence of cancer growing at another site. A high dose of steroids may allow for limited and transient neurological improvement. Radiotherapy should be decompressed on the spinal cord depending on tumour type and the clinical circumstances. There is a theoretical risk of radiation-induced oedema due to the fact that the spinal cord is even more sensitive to the effects of radiation than the brain. Radiosurgery with advanced devices may be an option for some patients. Patients with ISCM have a very short life expectancy. Median survival after diagnosis is made is 3 to 4 months and depends on both the type of tumour and treatment modality [8]. Regardless of treatment, many patients survive less than 1 year.

REFERENCES:

1. Greenberg S.M.: Handbook of Neurosurgery, ffifth edition(2001)Thieme Medical Publishner, New York NY 10001 US,pp.482-94
2. Hankey J.G.,Wardlaw M.J(2002)Clinical Neurlogy,Manson Publishing Ltd.London NW 11 DL,UK,pp. 567-72
3. Kaya Ar.,Dalkilic T.,Oser F.,Aydin Y(2003)Intramedullary Spinal Cord metastasis :A Rare and Devastating Complication of Cancer:Two Case Reports. Neurol Med Chir (Tokyo) 43:612-15
4. Gasser TG.,Pospiech J.,Stolke D.,Schwechheimer K(2001)Spinal Intramedullary Metastasis:Report of two cases and review of the literature.Neurosurgical Review 24(2-3):88-92
5. Villeges EA.,Guthrie HT(2004)Intramedullary Spinal Cord Metastasis in Breast Cancer:Clinical Feature, Diagnosis and Therapeutic Consideration :Case Report,The Breast Journal 10(6):532-35
6. Lindsay WK.,Bone I(2004)Neurology and Neurosurgery Ilustrated,Churchili Livingstone ISBN 0 443 07057 1,London,WIT 4LP UK,Fourth edition pp.386-97
7. Fredricks RK.,Elster A.,Walker FO(1989)Gadolinium enhanced MRI: a superior a superior technique for the diagnosis of intraspinal metastases.Neurology 139:734-36
8. Lee SS,Kim KM,Sym JS at al(2007)Intramedullary spinal cord metastasis: a single-institution experience.Journal of Neuro-Oncology 84(1):85-89

XXVIII CONGRESS OF THE EUROPEAN ACADEMY OF ALLERGY AND CLINICAL IMMUNOLOGY

Warsaw, from 6th to 10th June 2009

The 18th Congress of the European Academy of Allergy and Clinical Immunology was organized by the Congress Secretariat: Congress Sweden AB, Warsaw, from 6th to 10th June 2009. More than 1400 abstracts have been accepted, covering a wide range of topics. The venue for the EAACI 2009 Congress, the Palace of Culture and Science, is conveniently located at the heart of Warsaw. Over 6100 delegates participated in seven plenary sessions, over 80 scientific symposia, as well as Postgraduate Courses, Clinical Courses, Meet the Experts and Pro & Con sessions, spanning the whole period of the Congress and covering the whole area of allergology and related fields of clinical science, offered by more than 300 speakers from 42 countries. Over 40 companies exhibited at the Congress.

The opening of the Warsaw Congress honors the recipients of the EAACI Awards in 2009. The Clemens von Pirquet (Clinical Research) Award goes to Professor Stephen Durham, who is well known as an excellent researcher with a clear focus on the management of patients with allergy and asthma. He has put in immunotherapy on the international agenda of clinical science, and his follow-up study on the long-term effects of immunotherapy is famous. The Daniel Bovet (Treatment and Prevention) Award goes to Professor Bengt Björkstén for his pioneering work on gastrointestinal gut flora, contribution to ISAAC studies, and studies on risk factors for atopy-just some of the examples of the impressive record of accomplishment. Professor Rudi Valenta is the recipient of the Paul Ehrlich (Experimental Research) Award.

He has greatly increased our knowledge of and insight into the characteristics of allergens and allergenicity, and his immunotherapy studies with recombinant allergens are well known. The title of my abstract was ″Recommendations for use of FLC tests in monoclonal gammapathies″, and it has been accepted for poster presentation at the EAACI 2009. It was presented on Tuesday, June 09th at The Congress Hall Foyer. The posters were displayed in three groups and presented during “interactive poster presentation” sessions. The moderators selected the best posters for the “best poster awards”. Planning is already well underway for next year’s Congress in London, EAACI 2010, from 5th to 9th June 2010.

Vesna V. Radovic

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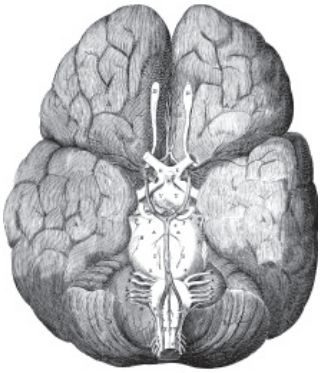

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