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MEDICINSKE ZNANOSTI

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ZAGREB, 2013.

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EDITORIAL

Dear readers, it is with great pleasure that I present to you RAD 39 (2013), the tenth volume of our journal, which has been continually published annually or semi-annually since 2006. I proudly announce that these 10 volumes of RAD obtained more than 62,000 hits on the web page "hrcak.srce.hr".

In the present volume, there are three excellent review articles written by the members of our Editorial Board (Vida Demarin, Iva Dekaris and Ivana Čepelak), and I cordially thank our distinguished colleagues for their contributions.

The present volume, in addition to the three mentioned regular review articles, introduces papers from the symposium entitled **"Epidemiological and Clinical Aspects of the West Nile Virus Infection in Croatia and the Neighbouring Countries"**. The Symposium was organized by the Department of Medical Sciences of the Croatian Academy of Sciences and Arts and held on 25 October 2012 at the Academy Palace. It was dedicated to the review of the recent progress in research on the virology, epidemiological, ecological and clinical manifestations of WNV infection, involving mosquitoes, birds, humans and horses.

We thank our guest editor, Prof. Josip Madić, Fellow of the Croatian Academy of Sciences and Arts, for his great effort regarding the compiling of the symposium papers. It is of particular importance that in the work of the Symposium, distinguished researchers from Austria, Croatia, Hungary, Italy, Serbia and Slovenia gave presentations and stimulated the discussion about selected topics. Unfortunately, some colleagues did not prepare articles for publication, and this is the reason why we have published only abstracts of these communications.

This volume further brings a report on the symposium entitled "Corneal Transplantation and Eye Banking", held at our Academy in January 2012. The report was written by the Chair of the Symposium, Prof. Iva Dekaris, associate member of the Croatian Academy of Sciences and Arts, to whom I am very thankful for the organization of this very successful international Symposium.

In your hands, dear readers, you are holding volume 39 of the journal RAD of the Croatian Academy of Sciences and Arts – Medical Sciences. I am very happy that our journal has managed to keep the continuity and join the relevant international databases. On behalf of the Editorial Board, I can promise you that we will continue in this sense in the future.

Marko Pećina

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Review article
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CURRENT TRENDS IN STROKE MANAGEMENT

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Summary

Stroke is a major health problem despite the great efforts made worldwide to fight against it. Despite therapeutic achievements to treat ischemic stroke patients in stroke units with tissue plasminogen activator (tPA), prevention remains the most powerful strategy to cure this complex disease. Stroke is a heterogeneous and multi-factorial disease caused by the combination of vascular risk factors, environment, and genetic factors. These risk factors can be modifiable or non-modifiable.

Recently, a great emphasis has been given to the investigations of genetic factors and stroke risk, which may lead to the discovery of new biomarkers for prevention, diagnosis and to the alternative strategies for stroke treatment. Ischemic stroke must be treated as an emergency. Immediate transportation to a nearest hospital, preferably a stroke unit, cautious lowering of excessive blood pressures (>220/120mmHg) and abstention from heparin and aspirin are the most important measures in the pre-hospital care for a stroke patient. Intravenous thrombolysis in a 3-hour window is the only approved treatment, but it is time-dependent. In severe strokes with occlusion of large intracranial arteries, mechanical recanalization is increasingly used. In order to improve stroke care in rural and urban areas where there is no organized stroke unit, it is useful to establish a stroke network, which functions according to telemedicine and teleneurology rules.

Keywords: cerebrovascular disease; ischemic stroke; stroke prevention; risk factors; stroke management; thrombolysis; telemedicine; teleneurology.

INTRODUCTION

The devastating stroke consequences have enormous personal, social and economic impact on oneself and the society. Stroke is the second leading cause of death worldwide. Its burden increases as the population ages and the incidence of the factors such as hypertension and diabetes increase across the globe [1]. Therapeutic strategies such as stroke unit care and treatments including tissue plasminogen ac-

tivator (tPA) have been developed to treat acute stroke more effectively and lessen the amount of disability that the disease carries [2]. However, these modalities are not available universally in developed countries and scarcely at all in developing ones, with t-PA utilization of less than 1 in 10 patients where it is even available [3]. More than 75% of strokes each year are first-ever strokes, making the primary prevention of utmost importance. Although stroke is a clinical diagnosis with many sub-classifications and distinct yet sometime overlapping entities, the identity of the risk factors is well known with many treatments readily available. The disease can be controlled, and perhaps largely prevented, thus achieving a sizeable public health benefit.

The stroke risk factors can be subdivided into non-modifiable (age, sex, race-ethnicity, genetic variations and predispositions) and modifiable (hypertension, diabetes, dyslipidemia, atrial fibrillation, carotid artery stenosis, smoking, poor diet, physical inactivity and obesity). An individual risk factor may contribute to each subclassification of stroke differently, and there is a large overlap or risk factors with cardiovascular and peripheral vascular disease. In this paper we will discuss the management of traditional and novel risk factors in stroke prevention, as well as management of stroke itself.

STROKE PREVENTION AND MANAGEMENT

Hypertension is the most important modifiable risk factor for stroke. Several studies have concluded that it accounts for more than the third of the stroke burden and maybe as much as half of all strokes [4]. The control of high blood pressure (BP) contributes to prevention of first strokes but also of renal and heart failure and possibly cognitive decline and frank dementia [5]. It has been shown that for every 20-mmHg increase in systolic and 10-mmHg increase in diastolic BP greater than 115/75 mmHg, there is a 2-fold increase in mortality associated with stroke and coronary disease [6]. Conversely, a 10 mmHg reduction in systolic BP has been shown to lower the stroke risk by about a third in primary and secondary stroke prevention [7,8]. These benefits also extend to the elderly, where in one study, a 36% reduction was found in the incidence of stroke for patients over the age of 60 who were treated with a thiazide diuretic with or without a beta-blocker. A more recent study of patients over the age of 80 showed that lowering the mean systolic BP by 15 mmHg and mean diastolic BP by 6.1 mmHg lowered the rate of fatal strokes by 39% after 2 years of treatment [9]. A meta-analysis of 31 trials, with 190606 participants, showed the benefits for educed BP in both younger (<65 years) and older (≥65 years), implying that the benefits from better pressure control can be reaped at any age [10].

A more intensive regimen appears to be more beneficial: in the ACCORD, a 5,000 patient study of those with diabetes, the patients who were in a more intense BP lowering group < 120, had a significantly lower risk of stroke after a follow-up of 4.7 years compares to those with a BP lowering goal of <140 [11]. While the BP lowering has reduced the risk for all stroke subtypes, these findings are more pronounced for hemorrhagic strokes.

A comprehensive evidence-based approach to treatment of hypertension is provided by the Eighth Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure [12]. Several categories of antihypertensive medications such as thiazide diuretics, b-adrenergic receptor blockers, angiotensin-converting enzyme inhibitors (ACEIs), angiotensin receptor blockers (ARBs), and calcium channel blockers (CCBs) have been shown to reduce the risk of stroke in patients who are hypertensive [10,13]. Thiazide-type diuretics were originally recommended as the preferred initial drugs of treatment for most patients. [14] A more recent meta-analysis, however, has shown that with a few exceptions (beta-blockers after a recent myocardial infarction and additional benefits of CCBs) all the different classes of BP-lowering medications produced a similar reduction in the incidence of stroke and cardiovascular disease for a given reduction in BP [15].

BP control can be achieved in a vast majority of patients, with most requiring combination therapies and often more than 2 antihypertensive medications. Unfortunately, BP is controlled in less than a quarter of the hypertensive population worldwide. Given the importance of hypertension as a stroke risk factor, and the abundance of effective treatments available, providing effective population-wide but patient-specific interventions remains a major public health care challenge.

Diabetes is an established risk factor for all vascular events in general and ischemic stroke in particular. Individuals with type 2 diabetes also have an increased vulnerability to atherosclerosis and an increased prevalence of hypertension, hyperlipidemia and obesity. Cardiovascular disease and ischemic stroke develops earlier in patients with diabetes, and strokes in patients with diabetes tend to have a heavier morbidity burden. American Diabetes Association recommends a multi-faceted approach to optimal health in diabetics; not only controlling the blood glucose, but also aggressive treatment of associated cardiovascular risk factors, with lower targets than for the general population. Surprisingly, recent studies have shown that aggressive treatment of blood glucose was very effective in preventing mircovascular complications of diabetes, but had no statistical effect on reduction of macrovascular events, including stroke [16,17]. However, the evidence that a multifactorial approach (reduced intake of dietary fat, light to moderate exercise, ce-

ssation of smoking) reduces stroke and cardiovascular risk in type-2 diabetics is supported by subgroup analyses of diabetic patients in large clinical trials. In the UK prospective Diabetes Study Group, comparing a tight BP control group (mean BP 144/82 mmHg) vs less stringent control group (mean BP 154/87 mmHg) resulted in a reduction of 44% of fatal and non-fatal stroke between the two groups. Another study found that adding a statin to existing treatments in high risk patients resulted in a 24% reduction in strokes. The Collaborative Atorvastatin Diabetes Study evaluated statin therapy in diabetic patients as a primary prevention of vascular events (18). A total of 2838 people with type 2 diabetes were enrolled, and the trial was stopped early due to its efficacy points being met: 37% reduction the primary vascular events in general, and a 48% reduction of strokes in particular [18].

Good glycemic control involves appropriate insulin therapy and professional dietary and lifestyle therapy for type 1 diabetics and weight loss, increased physical activity and, if need be, oral and injectable hypoglycemic agents for type 2 diabetics. Treatment of adults with diabetes, especially those with additional risk factors, with a statin to lower the risk of a first stroke is recommended. Studies have shown that a multi-faceted approach to controlling diabetes and concomitant risk factors leads to significant reduction in cardiovascular events and stroke.

Many epidemiologic studies found no consistent relationship between cholesterol levels and overall stroke risks. However, there is evidence that there is a positive correlation between total and low density lipoprotein (LDL) cholesterol levels and the risk of stroke. Conversely, high density lipoprotein (HDL) cholesterol levels have been associated with reduced risk of ischemic stroke across many sub-populations. Moreover, in high risk patients, lowering cholesterol with statins (HMG-CoA reductase inhibitors) has been shown to significantly reduce the risk of transient ischemic attack or non-cardioembolic stroke [19]. Several meta-analyses have shown that lowering the LDL cholesterol by 1.0 mmol/L reduced the risk of ischemic stroke by about 20% [20]. The beneficial role of statins for primary and secondary stroke risk reduction for those with high risk for cerebrovascular disease risk has been documented [18]. It has estimated that statins prevent 9 strokes per 1000 high risk patients or in those with coronary heart disease treated over the period of 5 years. Earlier concerns of statins increasing the risk of hemorrhagic stroke have not been substantiated by a recent meta-analysis, although the topic is still under debate and caution should be exercised [21,22].

The benefit of rosuvastatin in cutting the risk of myocardial infarction in half in those patients who were apparently healthy but had elevated levels of C-reactive protein hints at the many pleotropic effects of statins [23]. Although this class of drugs is very well studied, the way it protects the brain and the heart is not entirely

clear. It may decrease platelet aggregation, stabilize plaques, lower BP and reduce inflammation. There has been further speculation that they may have neuroprotective properties, improve endothelium function, decrease smooth muscle proliferation and increase the number of circulating endothelial progenitor cells. Intriguing results have shown statins to increase nitric oxide production and P-selectin expression and up-regulate tissue-type plasminogen-activator. It is unclear if statins lower the risk of stroke by lowering the LDL, or by any of the above and maybe yet-unknown mechanisms. Other surrogate markers for artherosclerosis, such as carotid intima-media thickness (cIMT), may prove to be useful in monitoring the progression of and treatments against stroke and other vascular diseases [24].

Non-statin lipid-modifying therapies may also offer stroke protection, although the studies are less equivocal. Niacin treatment has been shown to increase HDL as part of a combination therapy. Evidence has been mixed on the exetimibe/statin combinations and if they are superior to mono-statin therapies. Fibrates have been shown to decrease the risk of coronary events and retinopathy, but not that of ischemic stroke [25]. Fibrates, Niacin, exetimibe and omega-3 fatty acids each regulate serum lipids by different mechanisms and a combination therapy may be the final answer in achieving desired lipid control. National Cholesterol Education Program III [26] guidelines for the management of patients who have not had a stroke and who have elevated total cholesterol or elevated non–HDL cholesterol in the presence of hypertriglyceridemia have been endorsed in the US [26]. The updated clinical guidelines for cholesterol testing and management (ATPIV) from the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults were published at the end of 2012 [12].

Although the benefits of statin therapy outweigh the low risk of serious side effects, there are still some populations for which more data on the safety of lipid-lowering therapies are needed to clarify the risk associated with the effect of treatment, especially for older persons (>70 years of age) and women. More clinical trials and further research for optimal lipid-lowering strategies are needed as the complex relationship between dyslipidemia, atherosclerosis, stroke and cerebrovascular disease exists and has not been entirely elucidated.

Metabolic syndrome is defined by a cluster of interconnected factors that increase the risk of atherosclerosis, cerebrovascular disease, stroke, and diabetes mellitus type 2. Its components are dyslipidemia (elevated triglycerides and apolipoprotein B (apoB)-containing lipoproteins, and HDL), elevation of arterial BP and impaired glucose homeostasis, with abdominal obesity and/or insulin resistance [12,26,27]. More recently, other factors such as proinflammatory state, oxidative stress, and non-alcoholic fatty liver disease have been suggested to play an impor-

tant role in metabolic syndrome, making its definition even more complex. To date the most used definition for metabolic syndrome is the NCEP ATP III definition (26). All the components of metabolic syndrome are involved in conferring risk of stroke and cardiovascular disease. The adjusted hazard ratio (HR) for incident ischemic stroke associated with metabolic syndrome ranges between 2.1 and 2.5 in prospective studies, and a HR as high as 5.2 has been reported [28,29]. In a cohort of 14,284 patients, patients with metabolic syndrome but without diabetes exhibited a 1.49-fold increased risk of ischemic stroke or transitory ischemic attack, whereas those with frank diabetes had a 2.29-fold increased risk [29]. The relative odds for ischemic stroke or transitory ischemic attack, associated with presence of metabolic syndrome, were 1.39 in men and 2.10 in women. In NOMAS, a significant association between the metabolic syndrome and ischemic stroke risk was reported to be independent of other confounding factors including age, education, physical activity, alcohol use, and current smoking [30]. The prevalence of metabolic syndrome in NOMAS was 49%, and differed by sex (39% in men, 55% in women, $p<0.0001$) as well as race-ethnicity (56% in Hispanics, 41% in blacks, and 39% in whites, $p<0.0001$). Interesting, the effect of the metabolic syndrome on stroke risk was greater among women (HR=2.0; 95% CI, 1.3 to 3.1) than men (HR=1.1; 95% CI, 0.6 to 1.9) and among Hispanics (HR=2.0; 95% CI, 1.2 to 3.4) compared to blacks and whites.

Metabolic syndrome is also associated with subclinical atherosclerosis. In NOMAS, we have shown an independent association between metabolic syndrome and ultrasonographic subclinical measures of atherosclerosis including carotid plaque and carotid stiffness [31]. Therefore, an early identification of people at high risk for vascular accidents by evaluating subclinical markers of atherosclerosis is prudent in order to initiate preventive treatments.

Although the existence of metabolic syndrome as a separate entity has been recently questioned, individuals with a cluster of the risk factors that comprise metabolic syndrome should be aggressively treated for hypertension, dyslipidemia and diabetes. Patients with metabolic syndrome have a greater risk of stroke and other vascular diseases and therefore "*a major breakthrough related to the concept of the metabolic syndrome is the recognition of the high cardiovascular risk in subjects with a cluster of mild abnormalities or with a cluster of abnormalities that are not regarded as driving forces in cerebrovascular disease*" [32].

Atrial fibrillation is a common cardiac arrhythmia and a frequent cause of cardioembolic strokes. It account for up to 20% of all ischemic stroke, and the presence of atrial fibrillation independently increases the risk of these events by up to 5-fold [33]. The incidence of atrial fibrillation increases with age, with as many as 10% of the population experiencing atrial fibrillation in their 80s [34] and the number of

affected patients may reach 12 million just in the U.S. by 2050. Despite its increasing burden, atrial fibrillation is also arguably one of the best-studied causes of stroke with dozens of randomized trials and well-established evidence-based recommendations regarding effective medical treatments.

Stroke risk stratification models have been developed and validated. $CHADS_2$ (Congestive heart failure, Hypertension, Age, Diabetes, Stroke/TIA) is the most well-known stratification system [35]. It subdivides patients based on the independent predictors of stroke in those with atrial fibrillation and offers validated recommendations of anticoagulation vs antithrombotics therapy based on the scale scores. Several other models for predicting stroke risk, (such as the National Institute for Health and Clinical Excellence (NICE) guidelines and $CHADS_2$-VASc and bleeding risk (HAS-BLED) have since been developed [36].

Anticoagulation and antithrombotic therapies remain the main agents for stroke preventions for those with atrial fibrillation. Warfarin is the most commonly used anticoagulant that is cheap and exceedingly effective in preventing ischemic stroke: a recent meta-analysis showed a reduced risk of cardioembolic stroke of 64% for those on warfarin vs only 22% for those on aspirin. Warfarin also provides an almost 40% relative risk reduction compared to anti-platelet therapies [37]. Despite its effectiveness, this anticoagulant has several limitations (narrow therapeutic window, many drug and diet interactions, frequent and inconvenient monitoring) and has been under-utilized [38]. It is difficult to keep in range with only two-thirds of patients in clinical trials and little more than half in the community setting being in the therapeutic range.

Given the utilization gap for warfarin, several novel oral anticoagulants that are just as effective, have a better side effect profile and require less monitoring have been developed, tested and approved. The three novel oral anticoagulants that have shown the most promising effectiveness and safety data are Dabigatran [39], Rivaroxaban [40], and Apixaban [41]. They all exhibit a stable pharmacological profile, very few drug-drug interactions and are almost unaffected by the patients' diet. Very few patients (renal impairment or body weight extremes) require regular monitoring. They appear to be as effective, and in some cases superior to warfarin, with a much improved side effect profile. Less intracranial bleeding, arguable the most feared complication of coumadin, has been observed. These new agents will likely completely change how we treat patients with atrial fibrillation and lead to a greater reduction of cardioembolic strokes in the future [42].

Other times of cardiac disease that contribute to the risk of ischemic stroke include congestive heart failure, myocardial infarction, dialted cardiomyopathy, valvular heart disease (eg. mechanical valves, mitral valve prolapse, etc) and con-

genital defects [eg. patent foramen ovale, atrial septal defect and aneurysm]. All patients with prosthetic valves should be anti-coagulated. The rate of thromboembolism is reduced by half with antiplatelet therapy and by more than 75% with anticoagulation. Patients with congestive heart failure have a higher risk of stroke (2-3 fold) and are more likely to incur more significant stroke-related morbidity and mortality compared to those without heart failure [43]. Low ejection fraction (especially below <30%) has been identified as a risk factor for stroke, however, studies on the best treatments for this condition remain inconclusive. Presence of aortic arch atheroma is associated with increased risk of ischemic stroke. Congenital defects, while relatively common, contribute to the burden of stroke only in relatively specific circumstances. Most of these cardiac abnormalities and the potential thrombi that they produce require all and careful cardiac workup for detection, including a transthoracic and transesophageal echocardiography, and extensive cardiac monitoring with telemetry and often a more protracted outpatient cardiac event recorder.

Carotid stenosis of 50% or greater can be found in about 5-10% of people who are older than 65, and the prevalence of a severe asymptomatic carotid stenosis has been found in 3.1% of the population [44]. Data from observational studies and clinical trials indicate an annual risk of stroke attributable to extracranial carotid to have increased with the degree of stenosis (from less than 1% a year for a <80% stenosis to 4.8% per year for a >90% occlusive lesion). In Asymptomatic Carotid Atherosclerosis Study (ACAS), patients with asymptomatic carotid artery stenosis of ≥60% were randomized to carotid endarterectomy (CEA) or best medical management, with the results showing the primary outcome of ispilateral stroke, death or any perioperative stroke to be 5.1% for surgical candidate and 11% for patients treated medically over 5 years, with an absolute risk reduction of 1% a year [45]. Asymptomatic Carotid Surgery Trial (ACST) randomized asymptomatic patients with significant carotid stenosis (>60%) for immediate surgery vs. medical management and were followed for a mean of 3.4 years. The study found the overall 5-year risk of stroke or perioperative death to be 11.8% with deferred surgery and 6.4% with immediate endarterectomy. In the subgroup analysis, CEA appeared to be more beneficial for men than women, and in younger patients, more than older individuals. A more recent study, Asymptomatic Carotid Embolic Study (ACES), of patients who were followed for 2 years and had a asymptomatic carotid stenosis of at least 70% and were noted to have embolic signals found to have a significantly higher risk of ipsilateral stroke compared to those without any emboli, suggesting the detection of embolization on transcranial Doppler may be used for additional risk stratification [46]. The benefit of endarterectomy in asymptomatic stenosis is dependent on the surgical risk. Trials of carotid surgery for asymptomatic carotid disease reduced the

risk of stroke by about 1% per annum, while the perioperative stroke rate is 3%. Medical management should be offered to most patients and only high-volume centers with complication rate of ≤3% should contemplate the surgical procedure. It appears that men and those with life expectancy of more than 5 years will derive the most benefit in appropriate centers [47,46]. Most physicians, however, are not aware of CEA complication rates at their institutions. The best medical management has been evolving with wider use of antiplatelet agents, blood pressure and lipid lowering drugs, reducing the risk of stroke to 1% [48] and therefore the above relative benefit of CEA may need to be recalibrated.

Carotid angioplasty and stenting (CAS) was developed as a less invasive procedure compared to carotid endarterectomy. It has emerged as an alternative for patients who are high surgical risks, have many medical comorbidities, previous neck radiation, contralateral laryngeal nerve palsy or surgically-suboptimal anatomy. Since its invention over 20 years ago, the technique has evolved to more sophisticated and intricate stents, embolic protection devices and increasing operator experience. The Stenting and Angioplasty with Protection in Patient at High Risk of Endarterectomy (SAPPHIRE) Trial shows that stenting was non-inferior to CEA among high-risk surgical patients. The comparison of CEA and CAS has been extensively studied, often producing contradictory and confusing results. On one hand, multiple studies have shown that CAS is not as safe as CEA, especially in symptomatic patients, with the International Carotid Stenting Study (ICSS) being the latest addition to the mix. [49] On the other hand, Carotid Revascularization Endarterectomy versus Stenting Trial (CREST) found equal risk of composite primary outcome of stroke, MI or death in patients undergoing CAS or CEA [50]. The challenge of comparing three different modalities lie in the practice of modern medicine itself: the rapid evolution of medical management, CEA and CAS know how always slanting the risk-benefit ratio in a different direction. Overall, we have rapidly improving techniques for effective prevention of stroke from asymptomatic carotid stenosis.

Elevated plasma levels of homocysteine as a risk factor for stroke has been traditionally well recognized in atherothrombotic vascular diseases including stroke. It is believed that homocysteine induces endothelial platelet dysfunction, by reducing molecular nitric oxide. Folic acid and cobalamin have been shown to effectively reduce elevated homocysteine levels, however, clinical trials have failed to show that this translates into better cardiovascular or stroke outcomes [51,52]. Inflammation markers seem to be unaffected by lowering homocysteine in secondary stroke prevention, although it may have a role in patients with genetic predisposition to hyperhomocysteinemia or those who lack proper dietary folate intake.

Cigarette smoking is a well-established and modifiable risk factor for both ischemic and hemorrhagic strokes. Several meta-analysis have established cigarette smoking to impart a 2-fold increase in the risk of ischemic stroke an 3-fold increase of subarachnoid hemorrhages [54]. The most effective preventive measure is to not smoke or be exposed to smoke. Although quitting smoking is difficult to achieve, it does carry significant benefits, with rapid reduction in the risk of stroke within several years of cessation.

Alcohol consumption has been shown to have to have a J-shaped relation to risk of stroke, with light to moderate consumption (≤1 drink a day for women and ≤ 2 drinks a day for man) decreasing the risk of stroke to 0.3 to 0.5, but the risk increasing to 2 for heavier alcohol use (≥3 drinks) a day [54]. The relative risk is always increased for hemorrhagic strokes, regardless of the amount consumed. Alcohol in light to moderate quantities increases HDL cholesterol reduces platelet aggregation and lowers fibrinogen levels, while heavier use can lead to hypertension, hypercoagulability and atrial fibrillation [55]. Alcohol consumption should not be advocated as a way to prevent stroke, however, as alcoholism is a major public health problem and the risks of excessive intake remain great.

Abuse of illicit drugs such as cocaine in its various forms, heroin and amphetamines are associated with increased risk of both ischemic and hemorrhagic strokes by elevating the blood pressure and platelet aggregation, and inducing vasospasm and cardiac arrhythmias. Diet has been associated with the risk of stroke, with increased fruit and vegetable consumption having an inverse relationship to the risk of stroke in a dose-response manner; for example, for each serving per day increase in fruit or vegetable intake, the risk of stroke was reduced by 6% in one study. Research has shown that reducing salt intake improves cardiovascular and cerebrovascular health, although a recent review found no relation to salt intake and chronic heart disease morbidity and mortality [56]. Adherence to Mediterranean diet has also now proven to have a positive protective effect on cerebrovascular and cardiovascular disease [57].

Physical inactivity is another modifiable risk factor of stroke [58]. Physical activity has been shown to be beneficial in a dose-response pattern with more intensive physical activity providing greater benefits than light to moderate activity. The protective effects of physical activity are likely derived from lowering of body weight and BP and better glycemic control.

Obesity and body mass index (BMI) are risk factors for stroke, with associations to hypertension, dyslipidemia and glucose intolerance [59]. An obesity epidemic has been sweeping developed countries as well as developing nations such as India and China. The prevalence of metabolic syndrome worldwide, an entity that encom-

passes several stroke risk factors, was alarmingly high a decade ago (24-50%) and given the recent trends is likely to have increased since then [59]. Although no trials linking weight loss to the risk of stroke exist, evidence exists that losing weight reduces the presence of risk factors that cause stroke: in one meta-analysis an average weight loss of 5.1 kg reduced the systolic BP by 3.6-4.4 mmHg. Diet and exercise which are discussed above can be effective in controlling this modifiable risk factor.

Sleep related breathing disorders are common in patients with established cardiovascular disease. Habitual snoring and obstructive sleep apnea (OSA) have been shown to be independently associated with stroke and snoring has been strongly associated with vascular events during sleep. A recent-meta-analysis of 29 studies has shown that up to three-quarters of all patients have OSA, with the highest incidence of stroke occurring in patients with cryptogenic stroke, possibly establishing OSA an under-recognized stroke risk factor [60]. Hypoxemia, nocturnal hypertension and sympathetic surges have been postulated as some of many contributors to stroke in OSA patients. Decreased cerebral blood flow and impaired vasomotor reactivity has been observed even when the patients with OSA are not sleeping. Treatments with continuous positive airway pressure (CPAP) are non-invasive, and effective in reducing the risk of cardiovascular events and BP [61]. Further studies of OSA and other sleep disorders are on-going and may yield novels strategies and approaches in stroke prevention.

Aspirin has been shown as a well-established medication for primary stroke prevention. A recent meta-analysis showed a 32 % reduction in myocardial infarction in men but not women and a 17% reduction of the risk of stroke in women, but not men [62]. It is not clear why the sex difference exists, as the platelets seem to be inhibited equally in either sex, and no gender disparity was identified in studies in secondary prevention. A trial among diabetics with a history of atherosclerotic disease found Aspirin had no statistically significant effect on the rate of cerebrovascular events. Current guidelines indicate low-dose aspirin for women for whom the benefits may outweigh the risks and for patients with high CHD risk factors, but not for those at low risk or diabetics [63].

Stroke is a complex and multi-factorial disease caused by the combination of vascular risk factors, environment, and genetic factors. Recently, the scientific community put a great effort in understanding the genetic impact to the risk of stroke. Several epidemiological studies in families and twins have revealed a genetic component to stroke risk and experimental and clinical research using novel technologies have identified several genes directly or indirectly implicated in the mechanisms leading to stroke. The genetic contribution seems to be stronger in stroke patients younger than 70 years than in those who are older [64]. The strongest associations

have been found between stroke and single nucleotide polymorphisms (SNPs) in genes involved in inflammation, renin-angiotensin system, atherosclerosis, lipid metabolism, and obesity. (Matarin et al., 2010) However, few of these associations have been consistently replicated [65]. The innovation of a Genome-wide association study (GWAS) has allowed for identification of novel genetic loci without a specific hypothesis implicating a particular molecular pathway. The first GWAS for ischemic stroke was conducted using more than 400,000 unique SNPs in a cohort of 249 patients with IS and 268 neurologically normal controls [66]. However, these data did not reveal any single locus conferring a large effect on ischemic stroke risk. Other ischemic stroke GWASs have been conducted using a meta-analysis approach combining large populations such as CHARGE (Cohorts for Heart and Aging Research in Genomic Epidemiology), [67]. which consists of 4 prospective epidemiological cohorts of nearly 19,600 subjects with 1,544 incident strokes. In CHARGE, 2 SNPs were identified on chromosome (ch) 12, in the region of 12p13, and replication was obtained for one (rs12425791 SNP; the hazard ratio 1.3 for all stroke, and 1.0 for IS). A large International Stroke Genetic Consortium and the NINDS SiGN (Stroke Genetic Network) is currently conducting a GWAS of over 15,000 IS patients and 10,000 controls and expected to have the results available within a year.

Since stroke is a complex disease probably related to multiple genetic loci and the interaction of environment and heredity, the study of the precursors of this complex phenotype may be more rewarding. For example, the intermediate phenotypes as markers of subclinical disease such as cIMT, carotid plaque, arterial stiffness, and left ventricular mass; may be more helpful in identifying genes related to atherosclerosis and stroke [64]. The genetic research of stroke may greatly enhance our knowledge of this complex diseases. It may contribute to the discovery of new stroke biomarkers, which ultimately may be included in the stroke prevention, diagnosis, and treatment decisions.

When ischemic stroke does occur, the earlier stroke treatment starts the better the outcome. It is essential to minimalize the delay from symptom onset to therapy. Intravenous thrombolysis with alteplase within 3 hours is the only approved specific treatment of acute ischemic stroke. The benefit of thrombolytic therapy is time dependent [68,69]. For every 10 minutes of treatment delay, within 1-3 hours after stroke onset, 1 fewer patient out of 100 has a favourable outcome after systemic thrombolysis [69].

The majority of ischemic stroke patients unfortunately do not reach the hospital early enough to receive thrombolysis. For those patients, general acute stroke treatment is of utmost importance [70].

Prehospital stroke management starts with knowlede of stroke symptoms in general population, this being the cause of biggest delay in stroke treatment. Following the imporance of recognizing stroke, emergency department is crucial for successful stroke treatment [71]. It is usually the family members rather than the patients themselves contact emergency department. Informational campaigns distributed by mass media including TV' internet, social networks, and newspapers, provide education and encouragment to make an emergency call immediately after suspecting stroke occurence.

Apart from general public, emergency personnel and paramedics should also be continuously trained to improve preclinical stroke management [72]. Patients with facial droop, prior stroke or TIA and severe clinical deficit are most probable to receive a correct preclinical diagnosis of stroke, indicating that more subtle stroke symptoms may sometimes be misinterpreted or overseen [73]. The Face-Arm-Speech Test (FAST) is a simple acronim which can help identify stroke or TIA [74].

General practitioners should also be involved in preclinical stroke management, and participate in continuous training to be able to urgently evaluate every suspected stroke and channel the patient accordingly. Emergency department transportation ensures faster hospital admission due to quicker arrival, compared to private transport [75]. Whenever possible, transportation should be to the nearest hospital with a stroke unit. Prenotification to the emergency department and stroke unit physicians during transport ensures quicker in-hospital management and better chances to receiving thrombolysis.

During transport to the nearest hospital, patients should receive 0.9% saline intravenously. Heparin or aspirin should not be given before exclusion of intracranial hemorrhage by a CT scan. Hypertension can be tolerated up to 220/120 mmHg without lowering the BP. Oxygen should be supplied if saturation falls below 95% [76]. Upon arrival to the hospital the emergency department personnel should present the patient [73,75].

Immediate triage, neurological evaluation ircluding NIHSS, general physical examination, laboratory tests, and a native CT scan are needed [73]. Stroke or TIA mimics must be ruled out (including epileptic syncopes, sepsis [74], migraine with aura [77], or hypoglycemia). It is useful to implement in-hospital algorythms to speed up the evaluation and decision making.

Irrespective of age, gender, stroke subtype or stroke severity, admission to a stroke unit significantly reduces death, post-stroke dependancy, as well as need for institutional care after stroke. Stroke units are hospital wards with spacialized multidisciplinary staff trained for treating acute stroke and stroke related complications. Stroke teams consist of doctors, nurses, physiotherapists, occupational therapists,

speech and language therapists and social workers [78]. Technical equipment in a stroke unit allows assessment of neurological status, monitoring of vital parameters within 72 hours after severe stroke, ensure early mobilization and rehabilitation after stroke.

In most cases stroke is not acutely life-treatening, but majority of patients need stabilization of vital functions during the first hours to days after stroke. Treatment strategies aim at normalizing respiratory and cardiac functions, glucose, blood pressure, fluid balance, and at preventing stroke-related complications [76].

Stroke patients are at increased risk of cardiac arrhythmias, especially atrial fibrillation, myocardial infarction, heart failure or sudden death. Troponin levels can be elevated slightly during acute stroke, even in the absence of acute coronary syndrome, and thought to be due to stroke-related sympathoadrenal activation [79], making it necessary for every patient to have an ECG on admission. Further cardiac monitoring serves to maintain normal heart rates and can reveal paroxysmal atrial fibrillation as a common cause of stroke [76].

Hyperglycemia occurs frequently (30-40%; up to 60% in non-diabetics) in acute stroke and is associated with poor outcome and death, especially in patients without known diabetes [80]. Hyperglycemia was shown to be associated with hemorrhagic transformation of stroke and larger infarct volumes (81). However, it is uncertain if correction of elevated glucose levels improves clinical outcomes. To date, correction of glucose levels above 10 mmol/L with insulin, and below 2.8mmol/L with 10-20% glucose or dextrose bolus is recommended [76].

Hypertension is also common in acute ischemic stroke, and associated with increased risk of poor outcome. Due to impaired cerebral autoregulation during acute stroke, every change in systemic blood pressure directly affects cerebral blood flow. Hypertension may result in hemorrhagic transformation of the infarcted area, whereas hypotension may cause further damage to the penumbra. Despite such pathophysiological considerations, the optimal blood pressure management in acute ischemic stroke is not known. It is also unclear whether early discontinuation from preexisting antihypertensive treatment (about 50% of patients) is necessary [82]. Beneficial effects of early hypertension control could not be reproduced [83]. In the absence of conclusive data, current guidelines recommend moderate lowering of raised blood pressure over 220/120 mmHg, and over 185 mmHg systolic in thrombolysed patients using intravenous labetolol or urapidil [76], approximately by 15—25% during the first 24 hours atrial fibrillationter stroke [84]. Sublingual nifedipine has been described to cause abrupt decrease in blood pressure, and is therefore not a drug of first choice. In clinical practice, after permissive hypertension during the first 24 hours within the mentioned limits for nonthrombolysed and thrombolysed patients, antihypertensive medication may be continued or started from day 2.

Evidence how to handle hypotension is even more scarce. Low blood pressure at stroke onset is unusual and is recommended to be raised with saline 0.9% or volume expanders when associated with neurological deterioration. Inotropic support is only needed in patients with hypotension due to low cardiac output [76].

Oxygen should be supplied (usually 2-4 L/min via nasal tube) if saturation is below 95%. Saline 0.9% is recommended for fluid replacement during the first 24 hours after stroke. Fluid balance and electrolytes should be further monitored in dysphagic patients with severe deficit or impaired consciousness. Pyrexia (body temperature >37.5°C) should be treated with paracetamol and prompt the search for infections [76].

Bacterial pneumonia due to aspiration is one of the most frequent complications of acute ischemic stroke and should be treated with antibiotics. Aspiration occurs in patients with dysphagia or impaired consciousness and may be prevented by feeding by nasogastric tube, pulmonary physical therapy, and early mobilization [76]. Prophylactic antibiotic treatment, in contrast, may be harmful [85].

Urinary tract infections also commonly occur in hospitalized patients, mostly due to indwelling catheters. Almost half of stroke patients suffer incontinence at stroke onset (86), making urinary catheterization at least temporarily needed. Antibiotics should be used once urinary tract infection is diagnosed. Bladder catheters should be removed as soon as possible. However, 25% and 15% of patients will be incontinent at discharge and one year after stroke [86].

Immobilization due to paresis is a risk factor for deep venous thrombosis (DVT) and consecutive pulmonary embolism (PE). Early mobilization, rehydration and subcutaneous low molecular weight heparin can reduce the risk of DVT and PE in stroke patients without increasing the risk of hemorrhage [76,87].

Many stroke symptoms - hemiparesis, ataxia, vertigo, visual field defect, lower limb hypaesthesia, cognitive impairment, and depression - as well as polypharmacy lead to impaired gait balance and expose patients to increased risk of injury and falls. Hypovitaminosis D can be seen within one week after hemiplegic stroke. Falls occur in up to 25% of acute stroke patients, leading to serious injury, including hip fractures, in up to 5%. Physical exercise, mobilization, and supplementation of vitamin D, calcium, and biphosphonates can reduce fracture rates among acute stroke patients and should be provided in the acute setting. Drugs leading to postural instability, e.g. neuroleptics, should be avoided whenever possible [88-90]

Confusion, agitation and delirium are common problems in the acute phase of stroke. A search for underlying treatable causes often reveals dehydration, electrolyte dysbalance, fever, substance withdrawal, or nonconculsive epileptic seizures. When sedation or neuroleptics cannot be avoided, choice of drugs should take into

account potential side effects. Sedation can lead to impaired consciousness and thus increase the risk of aspiration and falls, so substances with short half-time periods, such as lorazepame, may be preferred. Antipsychotics, among them risperidone, have been associated with increased risk of cerebrovascular accidents in the elderly, risk of myocardial infarction in demented patients on cholinesterase inhibitors, and death [91,92]. The risk of cerebrovascular accidents seems to be greatest within the first weeks of drug intake, making the use of typical and atypical antipsychotics in the acute stroke setting even more hazardous. General recommendations are lacking, and prescription will be an individual decision based on comorbitidy and estimated harm if psychotic symptoms are left untreated.

Systemic thrombolysis with rt-PA within 3 hours is the only approved evidence-based therapy of acute ischemic stroke. Beyond 3 to 4.5 hours, intravenous thrombolysis remains effective and safe [68], but is yet unapproved by European medical authorities. Cerebral hemorrhage has to be excluded by CT scan before thrombolysis is started. As an off-label procedure, intravenous thrombolysis in the extended time window is routinely performed in experienced stroke centers. Data from the multicentre SITS-ISTR stroke registry showed that in 2009 there was a substantial increase (from 7% to 22%) in thrombolysis within 3 to 4.5 hours compared to 2008 [93]. However, the benefit of thrombolysis remains time-dependent (NNT in terms of a favourable outcome = 7 within 3 hours, 14 by 3 to 4.5 hours), [68]. Overall, the risk of SICH and mortality are slightly higher in patients thrombolysed within the extended time window, but the proportion of patients with favourable clinical outcome after 90 days is similar [93].

About 30% of strokes occur in people >80 years of age. Whereas approval criteria restrict thrombolysis to younger patients, it is now clear that older age is not a reason to preclude someone from treatment: risk and benefit must be weighted. Elderly stroke patients have higher bleeding rates. Mortality is also higher, but so is pre-stroke comorbidity. However, functional outcome in terms of mRS is significantly better in patients > 80 years after thrombolysis vs. without, and similar to younger patients [94,95].

In recent years, an increasing number of mechanical recanalization devices have been used to treat severe strokes with intracranial large artery occlusion as shown by CT angiography. The rates of good outcome (mRS = 0—2) in creased to 45 % with the latest techniques, rather acceptable for patients having very severe strokes [96]. For selection of patients the mismatch of cerebral blood flow and cerebral blood volume on contrast enhanced CT is used more and more instead of the time window. Perfusion CT has the advantage of being fast, widely available and less affected by artefacts than diffusion weighted and perfusion weighted magnetic resonance imaging.

Considering mentioned evidence that acute stroke patients benefit of specialized treatment in stroke units, but this specialized treatment is expensive and therefore not available everywhere. In most countries expertise in acute stroke treatment is mainly concentrated in academic hospitals, whereas a majority of stroke patients is treated in local general hospitals where the level of stroke care is partially suboptimal [97].

In order to improve stroke care in rural and urban areas it is useful to establish a stroke network. In order to take part in this kind of sophisticated stroke care, participating centers need to fulfill a number of requirements, such as: 24 hours availability of CT- or MR-imaging, Doppler-sonography, emergency laboratory diagnostics; securing a stroke care ward, with beds where all acute stroke patients of the hospital are concentrated, where monitoring of neurological status and vital parameters as well as early mobilization of the stroke patients is possible; continuous treatment by physiotherapists, occupational therapists and speech therapists; presence of a neurologist during the week and on call for emergencies during the weekend; the medical staff of the hospitals should be equipped with nurses, speech therapist and physiotherapist, as well as well as an additional physician [98].

These stroke teams have to complete a specific training program including: The training program is based on Standardized Optimized Procedures for diagnosis and treatment of stroke syndromes; Video training and certification in NIH-SS evaluation; Courses in transcranial Doppler sonography; Courses in swallowing disorders and dysphagia treatment.

A continuous stroke education program must include ward rounds in the local hospitals with one of the stroke experts every 3-4 months, newsletters and workshops is running in order to achieve further improvement of stroke care in the participating hospitals.

Therapeutic intervention in acute stroke requires urgent patient evaluation by physicians experienced in acute stroke to provide the best available care within critical time windows. Each hospital within the network should be equipped with a high-speed video conferencing system. In the local hospitals a second camera in a different position can be used. The interface of the local CT-scanners and MRI-scanners are connected to the workstation which is situated in a special room, close to the emergency facilities of the regional hospitals in order to facilitate rapid patient evaluation.

The stroke centers provide a 24-hours service with full-time stroke experts for teleconsultations. Contact is made up via telephone, the video connection is then established within minutes. The stroke specialist can download and analyze the CT or MRI-data from the server in the local hospital. Once the patient is in the exami-

nation room the physician at the stroke center can talk with him or with the local physician. The stroke specialist is visible on a screen in the examination room. The remote-controlled camera is operated by the stroke specialist in order to zoom and focus on the areas of interest. Mean duration of a video examination is between 15 and 20 minutes. Each teleconsultation is accomplished with a written report via electronic transmission. Indications to contact the stroke centers are clear cut [99].

CONCLUSION

Stroke remains a devastating and prevalent world-wide disease. The past several decades of research have also shown it to be a partially-preventable one, with many risk factors, strategies, and treatments identified, carefully evaluated and studied. A healthy diet and active lifestyle, careful control of modifiable stroke risk factors and access to regular health care are the keys to a successful stroke prevention strategy on both an individual and a public health level.

In stroke treatment evry minute counts. Raised stroke awareness within the population, rapid diagnosis by paramedics and primary care doctors, transportation by emergency deparment, hospital pre-notification and well organized in-hospital algorhytms contribute to rapid application of stroke treatment. The extended time window for systemic thrombolysis and recent data supporting thrombolysis in elderly patients >80 years of age offer the possibility that more patients receive this specific therapy for acute ischemic stroke. Acute general stroke management is best done at a multiprofessional stroke unit for 48-72 hours and deals with stroke-related metabolic, cardiorespiratory, inflammatory, and neuropsychiatric problems.

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Sažetak

Sadašnje smjernice za zbrinjavanje bolesnika s moždanim udarom

Unatoč konstantnoj borbi protiv moždanog udara u svijetu, on i dalje ostaje bitan zdravstveni problem. Iako je postignut velik napredak u liječenju moždanog udara primjenom aktivatora tkivnog plazminogena (tPA) u jedinicama za liječenje moždanog udara, prevencija ostaje najsnažnija strategija u liječenju te kompleksne bolesti. Moždani je udar heterogena i multifaktorijalna bolest uzrokovana kombinacijom vaskularnih čimbenika rizika, okoline i genetskih čimbenika. Na neke čimbenike rizika možemo utjecati, dok drugi ne ovise o nama.

Danas je naglasak stavljen na istraživanja genetskih čimbenika kao rizika za moždani udar. Ova istraživanja mogla bi dovesti do otkrića novih bioloških markera za prevenciju, dijagnostiku i alternativnu strategiju u zbrinjavanju bolesnika s moždanim udarom. Ishemijski moždani udar mora se zbrinjavati kao hitno stanje. Najvažnije mjere prehospitalnog zbrinjavanja bolesnika s moždanim udarom uključuju pravovremeni transport u najbližu bolnicu, ako je moguće u jedinicu za zbrinjavanje moždanog udara, pažljivo snižavanje izrazito povišenog krvnog tlaka (> 220/120 mmHg) te suzdržavanje od davanja heparina i aspirina. Intravenska tromboliza u trosatnom vremenskom prozoru jedino je odobreno liječenje, ali je vremenski ovisno. U slučaju teškog moždanog udara kod kojeg dolazi do okluzije velikih intrakranijalnih krvnih žila sve se češće izvodi zahvat mehaničke rekanalizacije. Kako bi se unaprijedila skrb za bolesnike s moždanim udarom u ruralnim i urbanim područjima gdje nema organiziranih jedinica za liječenje moždanog udara, korisno je uspostavljanje mreže za moždani udar koja funkcionira prema pravilima telemedicine i teleneurologije.

Ključne riječi: ishemijski moždani udar; prevencija; čimbenici rizika; zbrinjavanje bolesnika s moždanim udarom; tromboliza; rekombinantni tkivni plazminogen; telemedicina; teleneurologija.

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CURRENT TRENDS IN CORNEAL TRANSPLANTATION

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Summary

Corneal transplantation, as full-thickness transplantation, is an old surgical procedure which has been successfully performed for more than 100 years. However, in a last decade significant changes in surgical technique(s) have been implemented. The reason for such changes is the current trend to replace only the diseased part of the recipient cornea, not the whole cornea. Therefore, if the anterior part of the recipient cornea is opaque, the method of choice to treat such patient is anterior lamellar keratoplasty in which we can preserve the healthy recipient endothelium and thus decrease the chance of graft rejection. If the corneal disease involves posterior part of the recipient cornea, we use posterior lamellar keratoplasty, sparing the anterior part of the recipient cornea and thus improving speed and quality of visual recovery since the problem of high astigmatism caused by corneal sutures is solved. The advantages of lamellar transplantation are numerous: faster visual recovery, decreased rate of corneal graft rejection, preservation of the integrity of the globe, no suture-related problems (in case of posterior lamellar grafts), avoidance of complications related to "open-sky" surgery, theoretical possibility to use one donor cornea for two recipients and the use of local instead of general anaesthesia. Of course, there are also some disadvantages of such surgeries: increased early endothelial cell loss, detachment of the posterior lamellar grafts, vascular or epithelial in-growth into lamellar plane, and uncertain fate of lamellar grafts in a long follow-up. In this review the most frequently performed methods of corneal transplantation are presented, together with main advantages and disadvantages of different surgical techniques.

Keywords: anterior lamellar keratoplasty; superficial anterior lamellar keratoplasty; deep anterior lamellar keratoplasty; posterior lamellar keratoplasty; deep lamellar endothelial keratoplasty; Descemet stripping automated endothelial keratoplasty; Descemet's membrane endothelial keratoplasty.

INTRODUCTION

Penetrating keratoplasty (PK) or full thickness corneal transplantation has been a gold standard for the treatment of many corneal diseases for over a century, since

dr. Eduard Zirm performed the first successful PK in a human eye in 1905. Although PK in a low-risk corneal diseases had the best outcome regarding graft survival rate among all transplantations, clinicians soon became aware of some undesirable postoperative consequences of PK. These include: high astigmatism induced by the placement of corneal sutures causing prolonged visual rehabilitation despite the presence of completely clear corneal graft, unpredictable refractive outcome, and an increased vulnerability to eye trauma for many years after surgery. Therefore, many corneal surgeons posed themselves a logical question: why should we transplant an entire cornea and damage the structural integrity of the globe, when in many corneal diseases only one corneal layer is sick and need replacement? Already in 1950 dr. Jose Barraquer proposed lamellar transplantation of the posterior cornea (in which only the diseased part of the cornea is replaced by a donor tissue) in case of endothelial diseases, but due to the techniqual difficulties in performing lamellar transplantation it took time for this type of surgery to be widely accepted [1,2]. Since that time various techniques of lamellar transplantations had been utilized to treat corneal diseases at the proper location of the disease itself; meaning that only the anterior part of cornea is replaced if the diseases is present in the anterior corneal layers (anterior keratoplasty), or only the posterior part of the cornea if the corneal disease involves posterior part of the cornea – endothelium (posterior keratoplasty). In this review, the most widely accepted surgical approaches of lamellar corneal transplantations are presented.

ANTERIOR LAMELLAR KERATOPLASTY (ALK)

In this type of surgery the anterior diseased part of the cornea is replaced by a donor tissue, while posterior stroma and/or Descemet membrane and endothelium of the recipient cornea is preserved. Depending on the fact whether corneal disease involves only anterior stroma or gets deeper into stromal layers we can use two different surgical approaches: a) Superficial Anterior Lamellar Keratoplasty (SALK) if the disease involves only the superficial part of corneal stroma, and b) Deep Anterior Lamellar Keratoplasty (DALK) if we need to replace total or near-total corneal stroma. The main advantage of ALK is preservation of the healthy recipient endothelium and thus decreased risk of graft rejection, better preservation of structural integrity of the globe and decreased chance of intraoperative complications involved with „open-sky" procedures [3]. However, there are also disadvantages of such procedures such as interface scarring or „haze", residual corneal pathology, epithelial ingrowth and potential vessel ingrowth into the interface (in vascularised corneas) limiting patient's quality of vision.

Superficial Anterior Lamellar Keratoplasty (SALK)

The main indications for SALK are: stromal opacities located in the anterior stroma which may be caused by anterior stromal dystrophy (e.g. Reis-Buckler), degeneration (Salzmann nodular degeneration), infection, chronic inflammation or previous refractive surgery resulting in corneal scarring. Resection of the anterior diseased part of the recipient cornea can be performed by manual resection, with the help of microkeratome or femtosecondlaser [4,5]. If the femtosecondlaser is used to create the lamellar cut then the procedure is called femtosecond-laser assisted anterior lamellar keratoplasty or FALK. Depth of the anterior stromal opacity can be determined preoperatively by the use of anterior segment optical coherence tomography (OCT). Results obtained with manual resection are suboptimal due to the irregular interface and thus poor visual outcome, while better results are reported with microkeratome-assisted ALK [4]. After removal of the diseased part of the recipient cornea, a lamella of the same thickness is obtained from a donor cornea mounted onto the artificial anterior chamber, punched to the same size and sutured into the recipient bed. Reported complications of SALK are residual corneal pathology, haze, anisometropia, epithelial ingrowth and dry eye [5].

Deep Anterior Lamellar Keratoplasty (DALK)

The main indications for DALK are: deep stromal opacities which may be caused by herpetic or other infectious scars, chronic inflammation with scarring after corneal burns and keratokonus. In this procedure corneal surgeon aims to remove nearly all or all of the recipient corneal stroma, while preserving the healthy endothelium. The advantage of DALK is preservation of host endothelium and thus reduced incidence of graft rejection, faster visual rehabilitation as compared to PK, and lower incidence of serious complications such as expulsive haemorrhage or endophthalmitis. DALK was first described by dr. Anwar in 1972. when simple blade was used to dissect deep stromal layers from Descemet membrane (DM) [6]. The technique was further improved by Anwar and Teichmann in 2002 with so called „Anwar's big-bubble technique". In this technique 60-80% of stromal depth is removed and then an air-bubble is inserted deep into the stroma to dissect Descemet's membrane with endothelium from corneal stroma; carefully the remaining stroma is then dissected. Finally, donor graft without Descemet membrane/endothelium of same size or 0.25 mm oversized is sutured into place [7]. Although DALK brings very good visual and refractive outcomes and preservation of host endothelium, the techniques is not easy to perform and may be complicated with perforations of DM and consequent need for penetrating keratoplasty. The most common indication for

such surgery is keratoconus. Several studies have been made to compare postoperative results of DALK versus PK in keratoconus, and it has been shown that similar visual results will be obtained by both techniques; however it is important to bear in mind that in case of DALK recipient endothelium is preserved and thus the incidence of graft rejection is lower [8-11]. Therefore, in spite of the fact that DALK is more time-consuming and more difficult to perform as compared to PK, increasing number of corneal surgeons are choosing this technique for keratoconus patients in order to spare recipient endothelium in those mostly very young patients.

POSTERIOR LAMELLAR KERATOPLASTY (PLK) OR ENDOTHELIAL KERATOPLASTY (EK)

Posterior lamellar (PLK) or endothelial keratoplasty (EK) is the selective replacement of diseased endothelium with a healthy donor endothelium (either on Descemets membrane alone or together with a thin part of donor stroma). The most frequent indications for such surgery are: Fuchs dystrophy, pseudophakic bullous keratoplasty and decompensated corneal grafts. More and more surgeons are performing EK because it gives better results, faster visual rehabilitation and it is safer surgery compared to PK [12-17]. Endothelial keratoplasties are nowadays most widely accepted lamellar corneal transplantations; for example in a period between 2005 and 2008 the rate of EK in USA increased 10-folds, coming to the rate of 70% of all corneal grafts. The most widely performed type of EK in USA, called DSAEK, is representing 80% of all lamellar keratoplasties performed in that country today. In Europe, the number of EK is not as significant as in USA, but it is in a constant rise coming to the rate of 30% of all grafts in year 2011 (according to the data of European Eye Bank Association). As previously mentioned, dr. Jose Barraquer was the first to propose lamellar transplantation of the posterior cornea already in 1950, but his surgical technique was not widely accepted due to techniqual difficulties [1]. In 1998 dr. Melles invented a novel surgical technique for posterior lamellar keratoplasty. He had proposed that after stripping of the diseased recipient endothelium, donor lamella consisting of donor endothelium and thin stromal layer can be inserted through the small corneal opening, and then use an air-bubble to fixate the donor endothelial graft onto the recipient cornea (*Figure 1*). He had shown that the oedematous cornea can be cleared if provided with a new functioning endothelial cell layer via a posterior corneal graft [18-22]. In 2001, dr. Terry published results of „deep lamellar endothelial keratoplasty“ (DLEK) in first United States patients [23]. The procedure was also adopted by dr. Price who termed it „Descemet-Stripping with Endothelial

Keratoplasty – DSEK" [24]. At the beginning, lamellar cut of a donor cornea to obtain healthy donor endothelium was performed by manual lamellar dissection, but the lamellar interface was not smooth enough, so dr. Gorovoy started to perform lamellar cuts with an automated cutting system called microkeratome (usually used for LASIK in refractive surgery), and this procedure was named DSAEK (Descemets Stripping Automated Endothelial Keratoplasty) [25]. Later on, when the preparation method of donor posterior lamella became more standardized, many eye-banks trained their staff to do the lamellar cut in the eye bank and then deliver so called "pre-cut" donor tissue for DSAEK to the corneal surgeon. Most of the american corneal surgeons are using such a "pre-cut" tissue for their DSAEK cases, while in Europe DSAEK is still mostly performed in a way that surgeon prepares posterior corneal graft in the operating room prior to transplantation. From the clinical point of view, both methods of tissue preparation for DSAEK seam to perform equally. Advantages of DSAEK over penetrating keratoplasty are numerous: corneal astigmatism is much lower as compared to PK due to the lack of sutures (which are causing significant astigmatism in PK); therefore visual recovery is fast and most patients have usable vision within 6 weeks after operation, and some of them have excellent vision at just 1 week, especially with ultra-thin DSAEK grafts [26-28]. After PK the stable visual acuity does not occur for at least 6 months to a 1 year (*Figures 2 and 3*). Sometimes it takes even longer period and patients require hard contact lenses to help normalize their astigmatism; unfortunately many older patients find it difficult or impossible to wear lenses. One more advantage of all endothelial keratoplasties is that small incision that is made during the operation leaves almost entire thickness of the recipient cornea untouched. This results in normal tectonic strength of the eye with resistance to traumatic rupture for the rest of the patient's life, which is not the case in PK where a circular wound cuts out the entire corneal thickness. Consequently, this vertical and unstable PK wound never heals with significant strength and patients may have ruptured wounds and lose their eye from blunt trauma, even many years after PK. There are also disadvantages of endothelial keratoplasty such as detachment of the donor graft (which occurs most often with DMEK cases), and a question of endothelial cell density (ECD) loss which may lead to primary graft failure. Reported rates of primary graft failure after DSAEK are between 0% and 29%, and detachment rates are between 1% and 40% [28,29]. If detachment of the DSAEK graft occurs, the graft can almost always be re-attached by reinsertion of an air-bubble into the anterior chamber, but this means an additional surgery for the patient (*Figure 4*). ECD loss after DSAEK is usually between 24% and 40% at 6 months to 1 year, which is higher than the early cell loss reported in most recent PK series [29]. The early cell loss with DSEAK is not surprising because

it entails more donor tissue manipulation than PK. However, there is also a study showing that after 3-4 years endothelial cell loss was less in DSAEK than in PK [30].

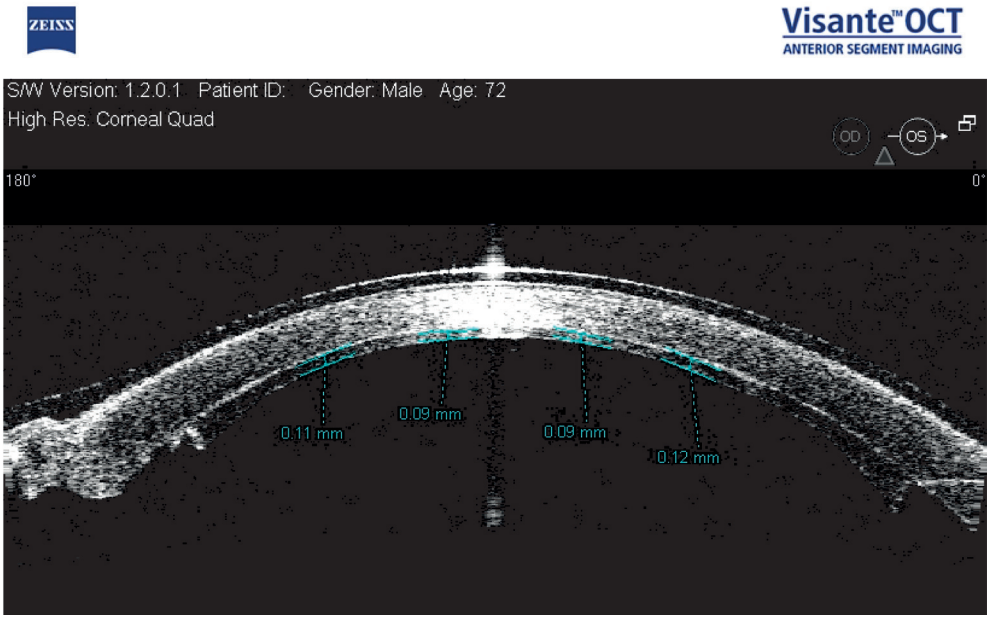

Figure 1. Posterior lamellar keratoplasty - anterior segment optical coherence tomography scan showing nicely adherent ultra-thin endothelial graft of 90μm thickness at first postoperative day.

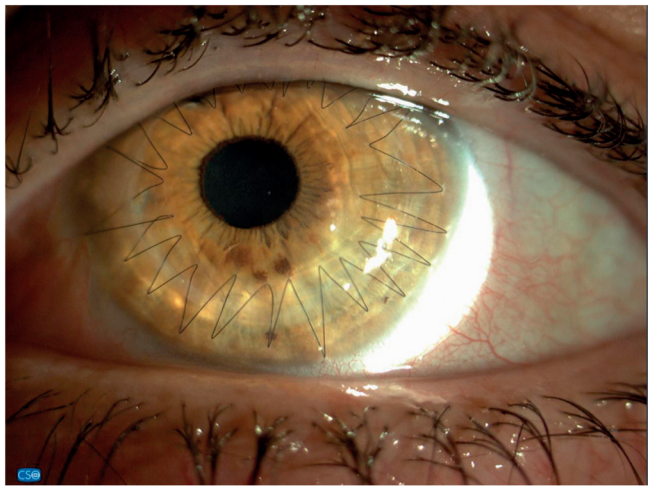

Figure 2. Clear corneal graft after penetrating keratoplasty at 6 months after surgery – uncorrected visual acuity is 50%, and best corrected visual acuity with a contact lens 100%.

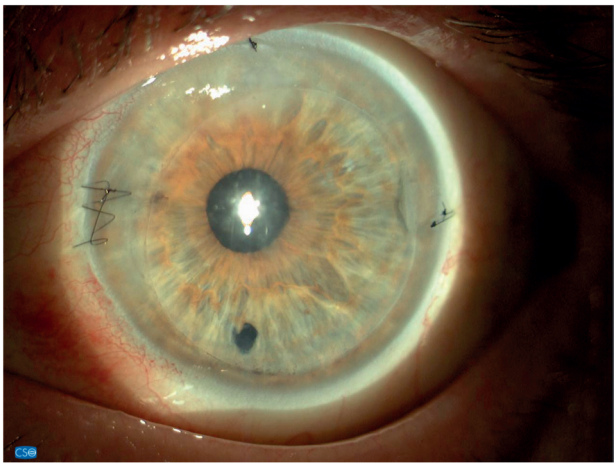

Figure 3. Endothelial graft after ultra-thin Descemet's Stripping Automated Endothelial Keratoplasty at 3 weeks after surgery – uncorrected visual acuity is 100%.

Figure 4. Posterior lamellar keratoplasty - anterior segment optical coherence tomography scan showing detached endothelial graft at three days after surgery. In such case it is necessary to place another air-bubble into the anterior chamber to reposition the graft.

Melles has investigated a further refinement of posterior lamellar transplantation and started with the transplantation of the Descemet's membrane alone; the procedure called Descemet's membrane endothelial transplantation or DMEK [31-34]. Main advantage of this procedure is that patients obtain better visual acuity in a quicker time-frame as compared to DSAEK, and that the graft rejection rate is significantly lower as compared to both DSAEK and PK [35,36]. However, the technique is technically more difficult with the reported graft detachment rate going up to 60%, and there is a general concern over higher endothelial cell density loss due to prolonged manipulation with such a thin donor graft. Price group made a prospective study comparing results of somewhat different approach - Descemet's membrane automated endothelial keratoplasty (DMAEK) with DSEK in 2011; study showed that DMAEK has a higher rate of postoperative air reinjections than DSEK and comparable 6-month endothelial cell loss [37]. In a recent study by dr. Kruse group in Germany it has been shown on a significant number of cases that DMEK provides faster and more complete visual rehabilitation when compared with DSAEK, without any significant differences concerning endothelial cell survival within a 6-month follow-up [38]. However, a long-time postoperative data on endothelial cell loss after DMEK are still lacking.

Having in mind that DSAEK is surgically much safer and easier then DMEK, and that the thinner endothelial grafts may bring quicker and better visual recovery [39-43], dr. Busin suggested so-called „ultra-thin DSAEK" as, in his opinion, currently optimal surgical approach for patients in need for endothelial lamellar transplantation [44]. The difference to conventional DSAEK is that ultra-thin donor tissue preparation for endothelial keratoplasty is made with a double-pass microkeratome; first cut is usually made with a microkeratome head of 250 to 350 μm and the second one with a 50 to 130μm head (depending on the thickness of the donor corneal tissue) [45]. This technique combines advantages of DSAEK (easier manipulation with the endothelial graft and consequently decreased endothelial cell loss) with the advantages of DMEK (thin grafts bring better vision) [46].

CONCLUSION

Corneal transplantation has changed dramatically since its early days over 100 years ago, when the gold standard of surgery was full thickness penetrating keratoplasty (PK). In a last decade many new surgical options have been proposed to treat patients with corneal diseases. The main standard of care nowadays is to remove only the diseased part of the recipient cornea and to replace it with a donor corneal lamella. The options for such a general idea are numerous, and it is up to a cor-

neal surgeon to choose an optimal surgical option for each individual patient. All the previously described surgical methods of lamellar corneal transplantation have their great advantages, but also their limitations that we must be aware of. Finally, the growing number of lamellar cases performed worldwide does not mean that PK becomes an obsolete technique, since there are still a significant number of patients having corneal diseases involving all corneal layers, and for which PK will remain the only way to regain their vision.

Abbreviations: PK- penetrating keratoplasty, ALK- anterior lamellar keratoplasty, SALK – superficial anterior lamellar keratoplasty, DALK – deep anterior lamellar keratoplasty, PLK- posterior lamellar keratoplasty, EK- endothelial keratoplasty, DLEK- deep lamellar endothelial keratoplasty, DSAEK - Descemet stripping automated endothelial keratoplasty; DMEK- Descemet membrane endothelial keratoplasty.

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Sažetak

Suvremeni trendovi u transplantaciji rožnice

Transplantacija rožnice vrlo je stara kirurška metoda koja se uspješno primjenjuje već više od 100 godina. U posljednjih desetak godina nastupile su velike promjene glede kirurških metoda koje se primjenjuju prilikom transplantacije rožnice. Razlog za tako drastične promjene jest suvremeni trend da se prilikom transplantacije zamijeni samo onaj oboljeli sloj primateljeve rožnice, a ne puna debljina rožnice. Takav kirurški zahvat naziva se slojevita ili lamelarna transplantacija rožnice. Ako je samo prednji dio bolesnikove rožnice zahvaćen bolešću, metoda izbora pri liječenju bit će prednja slojevita (lamelarna) transplantacija, pri kojoj se sačuva zdravi primateljev endotel, a time se i smanji šansa za odbacivanje transplantata. Ako je bolest rožnice zahvatila stražnji dio rožnice, primjenjuje se stražnja slojevita transplantacija kojom se postiže znatno brži oporavak i bolja kvaliteta vida negoli kod perforativne keratoplastike (PK) jer se izbjegava problem astigmatizma koji se nužno javlja kod PK radi postavljanja šavova rožnice. Brojne su prednosti slojevite transplantacije: brži oporavak vida, manja šansa za odbacivanje transplantata, očuvanost integriteta bulbusa, nema problema vezanih uz šavove rožnice (vrijedi za stražnju slojevitu transplantaciju), izbjegavanje komplikacija vezanih za rad na „otvorenom" oku, teoretska mogućnost uporabe jedne donorske rožnice za dva bolesnika (ako istog operativnog dana imamo bolesnika kojem radimo prednju lamelarnu i bolesnika predviđenog za stražnju lamelarnu transplantaciju) te mogućnost primjene lokalne (ili potencirane) anestezije umjesto opće anestezije. Naravno da postoje i problemi koji se mogu javiti kod takvih operacija, a to su: povećan rani gubitak endotelnih stanica rožnice, odljepljenje stražnjeg endotelnog transplantata, urastanje krvnih žila ili epitela u sloj između primateljeve i donorske lamele rožnice te nedovoljno informacija glede dugoročne sudbine lamelarnih transplantata. U ovom preglednom članku prikazane su danas najčešće upotrebljavane metode slojevite transplantacije rožnice, zajedno s njihovim prednostima i manama u odnosu na standardni PK.

Ključne riječi: prednja slojevita (lamelarna) transplantacija; površna prednja slojevita transplantacija; duboka prednja slojevita transplantacija; stražnja slojevita transplantacija, duboka stražnja slojevita transplantacija; automatizirana endotelna transplantacija uz ljuštenje Descemetove membrane; endotelna transplantacija Descemetove membrane.

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MAGNESIUM – MORE THAN A COMMON CATION

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Summary

Magnesium is an essential biological cation, participating in whole spectrum of biological functions. It is an irreplaceable factor for more than 300 enzymatic reactions, including those that use ATP as chemical energy source. Only about 1% of whole body magnesium is present in plasma. Normal plasma concentrations are in a range: 0.75-1.00 mmol/L.

Concentrations lower or higher than in this interval are called hypomagnesemia and hypermagnesemia, respectively. Those are life treathening conditions. Hypomagnesemia requires quick i.v. supplementation with magnesium cation. In addition to hypoamgnesemia, we are nowadays aware of a common "invisible" deficit of magnesium in tissues. This is a result of changing nutrition habits causing an insufficient recommended daily uptake (>300mg daily). Large clinical studies have shown that magnesium status is negatively correlated with incidence and severity of diabetes type 2, metabolic syndrome, hypertension and some arrhythmias. Therefore, food supplementation with magnesium is one of positive therapeutic or prevention options. Future larger clinical studies are expected to provide information on usefulness of supplementation in some other common diseases and syndromes (e.g. migraine, fibromyalgia, coronary artery disease, chronic fatigue syndrome).

Keywords: magnesium; hypomgnesemia; hypermagnesemia; therapeutic applications of magnesium.

INTRODUCTION

The fact that magnesium (Mg) is essential for life was for the first time described in animals by Leroy in 1926 [1] and Kruse et al. in 1933 [2], while the first description of clinical depletion of Mg in humans was reported in 1934 by Hirschfelder & Haury [3]. However, studies of Mg began to gain increased attention only since 1950, after

descriptions of various pathological conditions related to Mg deficiency [4]. Such studies were additionally facilitated by advances in technology, i.e. the development of ever more sensitive and specific methods for determining Mg concentrations in different samples so that more and more information has become available on regulation and biological functions of Mg at molecular level.

SOURCES, RECOMMENDED ALLOWANCES AND MAGNESIUM DISTRIBUTION IN THE BODY

Intake of Mg in the body occurs via drinking water (approximately 30 mg/L in hard water) and food. Mg is abundant in green leafy vegatables (chlorophyll rich and containing Mg), seeds, leguminous plants, cereals, hazelnuts [5,6]. Fruit, vegetables, meat and fish contain small amounts of Mg, while this mineral is least present in dairy food [7]. Mg is reduced by as much as 85% in refined or processed food [8], which accounts for a high prevalence of low Mg intake in the body.

It is well known that the entire quantity of ingested Mg is not absorbed in the body but only, on average, 30-50% [9]. To maintain serum Mg concentration within the reference interval (0.75-1.0 mmol/L) – which is the basis of Mg biological functions, Recommended Dietary Allowance (RDA) values established in 1997 [10] for daily Mg intake are >300 mg (310-360 mg for women and 400-420 mg for men) and they have been adjusted for age, gender and nutritional status (*Table 1*).

Table 1. Recommended dietary allowances (USA 1997) and reference intakes (Germany, Austria, Switzerland, 2000) for magnesium (mg/day) (reprinted from ref. 10)

Age	Women (mg/day)	Men (mg/day)
1-3	80	80
4-8	130	130
9-13	240	240
14-18	360	410
19-30	310	400
31-50	320	420
51-70	320	420
>70	320	420
Pregnancy	+40	
Lactation	+40	

Mg absorption occurs mostly in the small intestine, i.e. in the jejunum and ileum and significantly less in the colon [6]. Considering numerous factors that affect the process of absorption, the absorbed proportion of Mg is as stated above, commonly from 30% to 50% or 24-75% depending on body stores and food content [11]. The absorbed Mg is then distributed mostly to various tissue cells and to a lesser extent extracellularly. It is excreted through stool and through the kidneys.

Regarding its presence in the body, Mg is the fourth most frequent cation (following calcium, sodium, and potassium) [12,13]. From average 25 g (or 1120 mmol) of Mg in the body of an adult individual, the highest proportion of Mg can be found in bones, followed by muscles and other soft tissue cells [5,13]. One portion (20-30%) of Mg in bones is replaceable and buffers acute changes in serum Mg concentration but this quantity is significantly reduced with years [14,15]. Extracellularly, <1.0% Mg is found in three different forms: free ionized Mg, Mg complexed with different ligands, and as protein-bound Mg (*Table 2*).

Table 2. Distribution and/or concentrations of magnesium in a healthy adult (modified according to ref. 10).

Tissue	% total body magnesium
Bone	60-65
Muscle	27
Other cells	6-7
Extracellular	<1
Serum	0.75-1.1 mmol/L (55% free; 13% complexes with citrate, phosphate, etc; 32% bound to proteins, primarily to albumin)
Cerebrospinal fluid	1.25 mmol/L (55% free, 45% complexed)
Sweat	0.3 mmol/L (in warm environment)

PHYSIOLOGICAL FUNCTIONS OF MAGNESIUM

Magnesium is, after calcium, the second most frequent cation in cells [12]. 90% Mg in cells is bound to different ligands (e.g., nucleic acids, adenosine triphosphate, ATP, ADP, citrates, negatively charged phospholipids, proteins, etc.), while 10% Mg is in a free form. A significant amount of Mg is found in mitochondria (they are considered the major intracellular repository for Mg), then in the nucleus, ribosomes and endoplasmic reticulum [10]. In cells, Mg has structural and dynamic roles as, for instance, stabilization of protein structure, of phosphate groups in lipids of cellular membranes, of negatively charged phosphates of nucleic acids, and acti-

vation or inhibition of many enzymes [12,16,17]. Magnesium is actually important for the catalytic activity of more than 300 enzymes (e.g., ATP-ase, phosphofructokinase, enolase, adenylate cyclase, creatine kinase, 5-phosphoribosyl pyrophosphate synthetase, DNA polymerase, etc.), particularly of those that catalyze energy metabolism reactions. These reactions involve the process of glycolysis, Krebs cycle, respiratory chains, pentose phosphate pathway, gluconeogenesis, urea cycle, etc. In regulating enzyme activity, Mg can act as an allosteric modulator or as a co-factor, most frequently in the form of the Mg-ATP^{2-} complex [13,18]. Generation of a complex with ATP^{4-} is important to facilitate transphosphorylation reactions which are decisive for cell activation/deactivation. The complex is also involved in regulating the activity of ion channels that are significant for the transport of other electrolytes, e.g. potassium and calcium [19].

The biological role of Mg is rather heterogeneous. In addition to the above-mentioned structural and dynamic function, Mg – due to its relatively small atomic radius – easily competes with other divalent cations (particularly calcium) for specific binding sites on proteins [18,20,]. As an endogenous calcium antagonist, Mg is involved in, e.g., blockage of N-methyl-D aspartate (NMDA) receptor, inhibition of excitatory neurotransmitter release, blockage of Ca channels, and relaxation of vascular smooth muscle cells [21]. This characteristic, and that of binding to various ligands – particularly to ATP^{4-}, – is the basis for different physiological functions of Mg. Among other uses, Mg is essentially necessary for maintenance of normal neurological function and neurotransmitter release [6,8,13,22,23], muscular contractions/relaxations [6,9,24,25], regulation of vascular tonus and blood pressure [26,27, 28], of cardiac rhythm, [29,30,31], insulin signal transmission [32,33,34], parathormone secretion and activity [13,19,35], modulation of immunological functions [36,37], etc. It is therefore evident that tight regulation of Mg levels in blood /serum and Mg distribution to individual cell types are of vital importance.

MAGNESIUM HOMEOSTASIS

Although the importance of Mg in different physiological and biochemical processes is well known, direct hormonal control of Mg homeostasis has not been clearly described [38,39]. This is accounted for by Mg abundance in food during human evolution [10]. Indirect activity of hormones is mostly described at the level of intestinal absorption or renal tubular reabsorption. It is, actually, not known if hormone concentrations are under the influence of Mg status, which is the case when real hormonal control is present. The hormones in question are, e.g., calciotropic hormones (parathyreoid hormone, PTH, calcitonin and vitamin D) and, more rarely, insulin, glucagon, prostaglandins, epinephrine, aldosteron, etc. [40,41,42,43,44]. Mg

is, for instance, critical for maintenance of calcium homeostasis as it regulates PTH production and secretion, maintains adequate sensitivity of target tissues to PTH, is a cofactor of 1-alpha hydroxylase, an enzyme that participates in $1,25(OH)_2$ D_3 production, and also many calcium channels are dependent on Mg [45]. Besides its effect on calcium levels, Mg helps in regulation of other electrolytes (copper, zinc, potassium, sodium, etc.) so that the understanding of Mg homeostasis is important not only to understand disturbances in Mg levels but also for understanding and treatment of abnormalities of other electrolytes.

Magnesium homeostasis is controlled by dynamic interrelationships between intestinal absorption and by exchange with bones, but mostly by renal excretion [6,39,46].

Magnesium absorption

Mg absorption in the small intestine occurs primarily via non-saturable (passive) paracellular, and to a lesser extent by saturable (active) transcellular mechanisms [6,9,46,47,48].

The paracellular, passive mechanism involves absorption through small permeable spaces between epithelial cells (tight junction), and is responsible for 80-90% of Mg uptake in the body. This transport mechanism also includes some proteins from the claudin family (e.g., pracellin-1 or claudin-16) whose role is still at research stage [9,49]. Transcellular active transport to blood through the interior of intestinal epithelial cells is subject to tight regulation because ions must pass through apical and basolateral membrane. This mechanism includes specific channels (transient receptor potential channel melastatin - 6 and 7; TRPM-6 and 7) that are expressed on apical/luminal membrane of enterocytes [9,49,50,51]. It seems that the transport on basolateral membranes is associated with sodium gradient and corresponding activity of Na^+, K^+-ATPase [9].

Magnesium absorption in the intestines is generally dependent on the factors like the following: fiber rich food – phytates, organic acids, pH, Mg quantity, intestinal passage, meal volume and viscosity, vitamin D, calcium, phosphorus, polyphenols, oxalates, zinc, etc. [52,53,54,55,56].

Magnesium excretion

As serum Mg concentration is primarily controlled through urinary excretion, kidneys are considered the major regulators of Mg homeostasis. They have the ability to reduce Mg excretion to 0.5% of the filtered quantity in case of decreased serum concentrations, and to increase Mg excretion to 80% in case of elevated serum Mg concentrations [57,58].

Magnesium is filtered in glomerules (approximately 2400 mg/day), 90-95% of the filtered quantity is immediately reabsorbed, and only 3-5% is excreted, i.e. 100 mg/day [9,48]. To a lesser extent, reabsorption occurs via passive transport in proximal tubules (10-25%) [9], a significant proportion of 50-70% is reabsorbed in the thick ascending limb of the Henle's loop, and about 10% is reabsorbed in the distal part [6, 9, 59]. Magnesium reabsorption in the thick ascending limb of the Henle's loop occurs via passive paracellular pathway because of the electrochemical gradient that results in potassium exit through channels (renal outer medulla potassium channel, ROMK) in the apical cell membrane of the above-mentioned segment of the Henle's loop [60]. Reabsorption is at this stage further facilitated by claudin-16 (paracellin-1) and claudin-19 [50,61,62].

Ca^{2+}/Mg^{2+}- sensing receptor, CASR, that activates or inhibits Na^{+}-K^{+}-$2Cl^{-}$ co-transporter and ROMK channel on the apical side in conditions of hypomagnesemia or hypermagnesemia [63,64], is located on the basolateral cell membrane of the thick ascending limb of the Henle's loop and of the distal tubules.

Reabsorption in distal tubules is utilized to determine the final quantity of Mg that is to be excreted by urine. Reabsorption occurs via transcellular active mechanism that involves Mg entry into cells through the TRPM6 channel on the apical membrane (the role of TRPM7 channel is still investigated). It has been assumed that Mg exit from the cell on the basolateral membrane is facilitated, against concentration gradient, by the Na^{+}/Mg^{2+} dependent mechanism of exchange and/or Mg-ATPaze activity [64,65].

The processes of absorption and reabsorption are controlled by different regulatory factors. Thus, for instance, Mg intake through food and changes in TRPM6 expression play a role in regulating intestinal absorption [9]. Stimulation of absorption in the intestines with 1,25-dihydroxy vitamin D_3 ($(1,25(OH)_2D_{3)}$ was also described [66]. A regulatory factor of Mg reabsorption in kidneys is, e.g., epidermal growth factor (EGF) which regulates the activity of TRPM6 [67] whose expression is stimulated by estrogens [68].

Examination of Mg homeostasis at the level of cells themselves, i.e. Mg entry and exit from cells and its transport between cellular organelles via various pathways, carriers and exchangers, is in the focus of numerous studies that are currently underway. A reader is referred to an excellent review article by A.M Romani [12].

DISORDERS OF SERUM MAGNESIUM CONCENTRATIONS

Changes in serum Mg concentrations, related to reduced or increased levels outside the lower or upper limit of the reference interval (0.7-1.0 mmol/L), are termed hypomagnesemia and hypermagnesemia, respectively.

The occurrence of hypomagnesemia is more frequent, with the prevalence of 6.9% in general population and 7-11% among hospitalized patients [5, 69]. Hypomagnesemia in intensive care units ranges from 20% to 65% [70] and very frequently remains undetected. Some reports indicate a correlation between severe hypomagnesemia and increased mortality [71] so that it is recommended to monitor Mg concentration in severe patients.

Data on the prevalence of hypermagnesemia differ considerably and vary from 5.7% to 9.3% [13, 72]. Clinical findings related to serum Mg concentrations outside the reference interval are presented in *Table 3.*

Table 3. Clinical reports associated with altered magnesium concentrations (ref. 39).

Total Mg (mmol/L)	Report
<0.5	Tetany, convulsions, arrhythmias
0.5-0.7	Neuromuscular irritability
0.7-1.0	**Reference interval**
1.0-2.1	Typically without symptoms
2.1-2.9	Lethargy/listlessness, sleepiness, redness, nausea and vomiting, weakened reflexes of deep tendons
2.9-5.0	Drowsiness, weakened reflexes of deep tendons, hypotension, ECG changes
>5.0	Complete heart blockage, cardiac arrest, apnea, paralysis, coma

Symptoms of acute hypo- and hypermagnesemia partially overlap so that serum Mg concentration must be determined [6].

a) Hypomagnesemia

Hypomagnesemia and Mg deficiency in the body are the terms that are equally often used in practice, mostly referring to decreased serum Mg concentration. However, depletion of the total body Mg may be present with serum Mg concentrations being within the reference interval while, on the other hand, significant hypomagnesemia is possible without body Mg deficiency. In fact, the determination of the total serum Mg concentration is not the best method to evaluate Mg status in the body as there is very weak correlation between the levels of total serum Mg and total Mg status in the body.

Hypomagnesemia may be the consequence of decreased Mg intake, of Mg redistribution, and the consequence of extrarenal, renal and hereditary factors. Etiological factors of hypomagnesemia are summarily presented in *Table 4.*

Table 4. Etiology of hypomagnesemia (modified according to ref. 64*)*

Reduced Mg intake through food
Increased Mg loss via gastrointestinal tract: diarrhea, malabsorption, steathorrhea, small bowel bypass
Increased Mg excretion by kidneys: extracellular volume expansion, hypercalcemia, renal diseases, drugs (e.g. diuretics - thiazides, furosemide; amino glycoside antibiotics; cyclosporine; cisplatin inhibitors of proton pump, beta-adrenergic agonists, immunosuppressants, amphotericin B, foscarnet, etc.)
Hereditary causes: - Intestinal and renal: hypomagnesemia with secondary hypocalcemia (TRPM-6 mutations) - Renal: Bartter syndrome, Gitelman syndrome, familial hypomagnesemia with hypercalciuria and nephrocalcinose; autosomal dominant hypomagnesemia with hypocalciuria; isolated recessive hypomagnesemia, autosomal dominant hypocalcemia
Other etiologies: acute pancreatitis, alcohol induced tubular dysfunction, hungry bones syndrome, diabetes mellitus, enhanced sweating, increased requirements (pregnancy, growth)

Individuals with hypomagnesemia are often asymptomatic as Mg deficiency is usually secondary to other disease processes or drugs so that primary disease disguises Mg deficiency.

The signs and symptoms of hypomagnesemia depend more on the rapidity of decline in Mg levels than on serum Mg concentration itself [13], and they are usually not observed until serum Mg level drops below 0.5 mmol/L or lower. Clinical and biochemical manifestations of hypomagnesemia include the most frequent disorders of other electrolytes, disorders of cardiovascular, neuromuscular and central nervous system, complications due to Mg deficiency like hypertension, osteoporosis, glucose homeostasis disorder, atherosclerotic vascular disease and, e.g., migraine, asthma, etc.

Development of hypocalcemia in hypomagnesemia is associated with the role of Mg in the synthesis, secretion, and activity of parathormones on target tissues, and with the activity of $1,25(OH)_2D_3$. [73]. A significant proportion of individuals (40-60%) with hypomagnesemia also have hypopotassemia [72], and the mechanism involves the following: a) concurrent loss of Mg and potassium due to diarrhea or application of diuretics, and b) decline in the activity of Na^+, K^+-ATPase, that is, increase in the number of open potassium channels in the cells of the thick limb of the Henle's loop and collecting tubules [74, 75]. The hypopotassemia associated with hypomagnesemia is often refractory to potassium treatment so that Mg deficiency should also be corrected. Due to impaired electrical activity, myocardial

contractility and vascular tonus, cardiovascular disorders include, for instance, hypertension, dysrhythmias, ECG changes (e.g., prolonged QT interval, prolonged PR interval), atherogenic dyslipidemia, oxidative stress, impaired coagulation process, aggravated inflammatory problems, etc. [76,77,78].

Neuromuscular disorders, or neuromuscular hyperexcitability, are frequent in individuals with hypomagnesemia and involve, e.g., tetany, spontaneous carpal-pedal spasm, vertigo, ataxia, muscular weakness, convulsions, nystagmus, and psychiatric disorders like depression and psychosis, etc. [77,79]. The mechanism of the development of neuromuscular disorders is related to decreased axon stimulation threshold and increase in nerve conduction velocity due to reduced Mg concentration. Besides, as Mg participates in neurotransmitter (glutamate) release at neuromuscular junctions [80] and thus inhibits the calcium entry into presynaptic nerve terminals, enhanced calcium entry occurs in the situation of Mg deficiency and consequently of increased neurotransmitter release, which results in enhanced neuromuscular activity. Hypocalcemia is often concurrent with hypomagnesemia and it further contributes to neurological signs.

In case of inherited types of hypomagnesemia, whose molecular mechanisms have been discovered during the past ten years, they involve gene mutations for different proteins - channels, carriers, transporters [9,48,49], some of which are presented in *Table 5.*

Table 5. Some proteins of molecular magnesium homeostasis are related to hereditary hypomagnesemia.

Protein	Gene	Function	Comorbidity
Claudin-16 (ili paracellin-1) Claudin-19	*CLDN16* *CLDN19*	Allowability of paracellular permeability	Familial hypomagnesemia with hypercalciuria and nephrocalcinosis
NCCT	*SLC12A3*	(Na^+ Cl^- cotransporter)	Gitelman syndrome
TRPM-6	*TRPM6*	Selective Mg channel	Hypomagnesemia with secondary hypocalcemia
Na^+, K^+ -ATPase gamma subunit	*FXYD2*	Altered kinetics of Na^+ i K^+ exchange	Isolated dominant hypomagnesemia with hypocalciuria
EGF	*EGF*	Enhanced TRPM-6 activity	Isolated recessive hypomagnesemia
NKCC, Barttin, CIC Kb, ROMK	*SLC12A1,BSND, CLCNKB, KCNJ1*	Na+, K+ -cotransporter, Barttin, CIC-Kb-channel, ROMK-K-channel	Bartter syndrome

Hypomagnesemia therapy includes oral Mg replacement in food or food supplements in mild cases [39, 81] while intravenous replacement is applied in severe hypomagnesemia, most frequently in the form of Mg- sulfate [8, 82].

b) Hypermagnesemia

Hypermagnesemia is a condition where serum Mg concentration is >1.0 mmol/L. Mild hypermagnesemia is quite common in hospitalized patients and is usually not associated with clinical symptoms. Significant hypermagnesemia is related to different neuromuscular and cardiovascular disorders, and very severe hypermagnesemia may result in a coma.

One of the causes of hypermagnesemia is increased oral, rectal or parenteral intake of high Mg doses as, e.g., Mg supplementation in the form of magnesium salts or Mg-containing drugs (antacides, laxatives, purgatives), in some therapeutic approaches involving intravenous Mg application (e.g., torsade de pointes therapy of specific types of arrhythmia or convulsions in patients with eclampsia). Another cause is renal function impairment (acute and chronic diseases, rhabdomyolysis) because kidneys are the main organ that maintains Mg homeostasis (normal Mg concentration is maintained until glomerular filtration rate drops below 30 mL/min [13]. In such cases, renal compensatory mechanisms to maintain Mg homeostasis become inadequate and hypermagnesemia develops. Other causes include Mg redistribution to cells and, e.g., lithium therapy, familial hypocalciuric hypercalcemia, theophylline toxicity, etc. [39,83,84].

Signs and symptoms of hypermagnesemia occur at different Mg concentrations but they usually occur when serum concentrations are >2.0 mmol/L [13]. However, cases with very high serum Mg concentrations, i.e. 13.0 mmol/L [85], 17.0 mmol/L [86], 21.5 and 22. 5 mmol/L, have been described in the literature [87].

Clinical manifestations of hypermagnesemia include the most frequent neuromuscular and cardiovascular disorders (Mg is cardiotoxic in serum concentrations >3 mmol/L), nausea, and vomiting (*Table 6*).

Table 6. Clinical manifestations of hypermagnesemia (reprinted from *ref.13)*

Neuromuscular manifestations: confusion, lethargy, respiratory depression, absent tendon reflexes, paralytic ileus, bladder paralysis, muscle weakness/paralysis
Cardiovascular manifestations: hypotension, bradycardia, inhibition of AV and intraventricular conduction, heart block, cardiac arrest
Others: nausea, vomiting

With regard to biochemistry disorders, hyperpotassemia and hypocalcemia also occur in addition to hypermagnesemia [13,39].

Hypermagnesemia therapy involves discontinued Mg application and Ca-gluconate administration, while severe types of hypermagnesemia may even require hemodialysis [7].

CLINICAL ASPECTS

Physiological function of Mg in the body are numerous and varied (modulation of transport functions, of enzymatic activities, energy metabolism, synthesis of proteins and nucleic acids, etc.). It is, therefore, not surprising that Mg deficiency is involved in pathogenesis of a large number of pathological conditions (*Table 7*), with concurrent accumulation of data on the benefits of supplementing Mg as a pharmacological compound.

Table 7. Literature review of some pathological conditions related to magnesium deficiency

Pathological condition	References
Diabetes mellitus	24, 28, 88, 89,90,91,
Metabolic syndrome	92,93,94,95,
Cardiovascular diseases	10, 24, 48, 77, 96,97,
Arrhythmias	98, 99, 100, 101,
Hypertension	28, 98, 102, 103, 104, 105, 106,
Acute myocardial infarction	13,98, 107,108,109,
Atherosclerosis	13, 98, 110,111
Cerebrovascular disorders	71, 112,113,114,115,116,117,118
Headache (migraine)	119,120,121
Neuromuscular disorders	6,13,77,122,123,124,
Osteoporosis	13, 125,126,127,128
Asthma	13,77,129,130,131,
Preeclampsia/eclampsia	6,77, 132,133,134,135,
Malignant diseases	6,71,136,137,
Fibromyalgia	138,139
Cataract	140,141,
Pheochromocytoma	142,143,
Allergies	8, 144, 145, 146, 147,148,
Oxidative stress	149,150,
Drug application	6, 151,152,153,154,155,156

Early recognition of disorders of Mg metabolism (and its consideration with changes in other electrolytes) and their correction are necessary to avoid complications related to cardiac arrhythmia, hypocalcemia, etc. It is therefore necessary to frequently determine Mg in serum and possibly start Mg supplementation. Currently, different methodological approaches are available to estimate Mg status: methods for determination of total serum Mg concentration [6, 13, 77, 157], of ionized Mg form in whole blood, serum or plasma [13,48, 158], methods for Mg determination in erythrocytes [159], leukocytes [160], and skeletal muscle cells [25, 77, 161]. Magnesium may also be measured in urine, saliva, hair, and teeth [6,159]. In some clinical conditions, physiological tests are used as, e.g., Mg balance test, Mg excretion in 24-h urine, Mg loading test, Mg tolerance test [13, 162]. Recently, dry chemistry methods have also been developed for determination of ionized Mg for point-of-care testing [163]. Furthermore, for determination of ionized Mg concentration in the cell cytosol, the procedures with different metalochromatic or fluorescent dyes are used, as well as Mg-selective electrodes, and nuclear magnetic resonance and isotope testing [13, 48] which are mostly limited to scientific investigations.

Regarding therapy, Mg is usually administered in case of constipation [164] and dyspepsia [82]. According to reports by Guerera MP, et al. [82] and Geiger H, et al. [98] from 2012, indications for therapeutic applications of Mg (it has shown to be highly effective when applied intravenously) are primary reduction of the risk for eclampsia in preeclamptic women, arrhythmias (torsade de pointes in patients with long QT syndrome and digoxin induced arrhythmia), and severe asthma and migraine attacks.

Favorable effects of Mg have been observed in the control of glycemia in type 2 diabetes, improved efficacy of antihypertensive therapy, reduction in the frequency of muscular cramps, favorable effects on some risk factors of atherosclerosis, prevention of osteoporosis, of renal stone recurrence, of stroke, etc.

Although oral Mg supplementation is generally well tolerated, the following side-effects have been observed: nausea, vomiting, diarrhea, whereas large amounts of magnesium may result in hypotension, muscular weakness and coma. Mg must not be administered as therapy to patients with any type of renal dysfunction.

CONCLUSION

It is well known that Mg is the essential element for human health. However, due to increasing intake of Mg-deficient processed food, Mg deficiency may be expected to develop into a significant public health problem. It is not uncommon that hypomagnesemia is already a relatively frequent disorder which is, moreover,

often not recognized/not diagnosed because Mg concentration is seldom routinely determined. Actually, the evaluation of the Mg status is made difficult by the fact that most Mg is stored in tissues so that serum Mg levels are not representative. Generally accepted attitude is, however, that physicians should request Mg concentration measurement regardless of this difficulty, particularly in patients with the risk of possible hypomagnesemia.

As the low Mg intake may result in various, even severe, diseases, or contribute to complications that accompany different illnesses, it is necessary that researchers have thorough knowledge of those conditions in order to be able to assess symptoms related to Mg deficiency. In this regard, the application of novel analytical procedures in determining total and ionized Mg in different body fluids and cells, numerous clinical observations, and molecular genetic studies will certainly contribute to elucidation of the Mg role in terms of physiology and pathophysiology and particularly with regard to Mg deficiency treatment.

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Sažetak

Magnezij – više od običnog kationa

Magnezij je kation koji je vrlo bitan, nezamjenjiv, za brojne aspekte biologije stanice. Ta se tvrdnja najbolje zrcali u činjenici da je nezamjenjivi kofaktor za više od 300 enzimskih reakcija, uključujući najveći broj onih koje upotrebljavaju ATP kao izvor energije. Samo oko 1% ukupnog magnezija u organizmu čovjeka nalazi se u plazmi. Normalne koncentracije kreću se od 0.75 do 1.00 mmol/L.

Smanjena, odnosno povećana, koncentracija magnezija u plazmi naziva se hipomagnezijemija, odnosno hipermagnezijemija. Hipomagnezijemija može biti za život opasno stanje koje treba brzo sanirati povećanim (i.v.) unosom magnezija. Danas je poznato da, uz kliničku hipomagnezijemiju, vrlo često postoji i „nevidljiv" deficit magnezija u tkivima. Najčešće se javlja kao posljedica moderne prehrane, koja često ne zadovoljava dnevne preporučene doze (> 300mg dnevno). Kliničke studije pokazuju da su bolesti poput metaboličkog sindroma, dijabetesa tipa 2, nekih aritmija te hipertenzije, u pogledu pojavnosti i težine obolijevanja, u obrnutoj korelaciji s magnezijskim statusom organizma. Stoga je dodavanje magnezija prehrani jedna od mogućnosti pozitivnog utjecaja na spomenute bolesti. Od budućih kliničkih studija očekuje se jasna procjena opravdanosti magnezija kao dodatka prehrani i u nekim drugim bolestima i sindromima (migrena, fibromijalgija, bolest koronarnih arterija, kronični sindrom umora).

Ključne riječi: magnezij; hipomagnezijemija; hipermagnezijemija; terapijske aplikacije magnezija.

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Symposium

EPIDEMIOLOGICAL AND CLINICAL ASPECTS OF WEST NILE VIRUS INFECTION IN CROATIA AND NEIGHBOURING COUNTRIES

Guest Editor

Josip Madić

PREFACE

Although specific antibodies to West Nile virus (WNV) in human beings, horses and European brown bears had been previously detected, the first laboratory-confirmed human cases of the West Nile virus neuroinvasive infection in Croatia were diagnosed in September 2012 in three eastern Croatian counties. That outbreak of the WNV infection prompted us to organize the symposium under the title **"Epidemiological and Clinical Aspects of the West Nile Virus Infection in Croatia and the Neighbouring Countries"**. The Symposium was organized by the Department of Medical Sciences of the Croatian Academy of Sciences and Arts and held on 25 October 2012 at the Academy Palace. It was dedicated to the review of the recent progress in research on the virology, epidemiological, ecological and clinical manifestations of WNV infection, involving mosquitoes, birds, humans and horses. Distinguished researchers from Austria, Croatia, Hungary, Italy, Serbia and Slovenia gave presentations and stimulated the discussion about selected topics. Regretfully, not all papers of all presentations are included in this issue.

Until 2012, WNV infections in Croatia have never been associated with clinical symptoms. The clinical manifestations of WNV infection in humans from the wetlands of northeastern Croatia, foraging and nesting habitats for many wild birds species and mosquitoes, are described in the presented articles. In addition, IgM seropositive horses indicate the active focuses of WNV in Croatia, and might suggest a possible role of these animals as sentinels for human risk due to WNV. Although the vast majority of WNV infections are acquired from the bite of infected mosquitoes, it is necessary to emphasize that WNV can be transmitted through blood transfusion, organ transplantation, transplacentally, and probably through breast milk.

All these aspects were elucidated at the Symposium. It may be concluded that the expanded knowledge about WNV illness in humans seeks a new platform for a future implementation of diagnostic test and therapy of the disease in Croatia.

The organizers of the Symposium wish to express their gratitude to the Editor-in-Chief of the journal *Rad*, Marko Pećina, Fellow of the Croatian Academy of Sciences and Arts, for granting them the opportunity to publish the papers in this issue.

On behalf of the Organizing Committee,

Josip Madić

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HUMAN WEST NILE VIRUS INFECTION IN EASTERN CROATIA, AUGUST-SEPTEMBER, 2012

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Summary

In the period from 6 to 21 September, Croatia reported the first clinical cases of human West Nile virus (WNV) neuroinvasive infection in three counties (Brod-Posavina, Osijek-Baranja and Vukovar-Srijem County). The first case was most probably imported from Serbia where the patient resided during the incubation period, while the others represent autochthonous cases. Five patients presented with meningoencephalitis and two with meningoencephalitis followed by acute flaccid paralysis. In all samples WNV IgG and/or IgM antibodies were detected using enzyme-linked immunosorbent assay (ELISA) and confirmed by plaque-reduction neutralization (PRNT) and micro-neutralization test (MNT). Following the outbreak, a total of 178 serum samples were collected from healthy inhabitants of Osijek-Baranja County where the incidence of cases were highest. WNV IgG antibodies were detected by ELISA in 4 (2.2%) participants indicating the increased WNV circulation in this area in comparison with season 2011 resulting in the first reported clinical cases.

Keywords: West Nile virus; outbreak; seroprevalence; Croatia.

INTRODUCTION

West Nile virus (WNV) is a small, enveloped virus that belongs to the genus *Flavivirus* of the family *Flaviviridae* [1]. The virus is maintained in an enzootic cycle between *Culex* mosquitoes and birds, whereas humans and horses are considered dead-end hosts due to low-level and transient viremia [2]. However, humans may occasionally transmit or acquire virus in utero, through breast milk, via blood transfusion or organ transplantation, or through occupational exposure [3]. Most human infections (~80%) are asymptomatic. Approximately 20% of infected persons develop a non-specific febrile illness ("WNV fever"). A small number of symptomatic cases (<1%) progresses to the neuroinvasive form of infection characterized by meningitis, encephalitis and acute flaccid paralysis [4]. The epidemiology of WNV is continuously changing and currently the virus has a wide geographic distribution. After the first isolation of WNV in Uganda in 1937, few outbreaks in humans or horses were recorded until the beginning of 1990s [5]. A series of outbreaks in the 1990s (Algeria 1994, Morocco 1996, Romania 1996, Tunisia 1997, Italy 1998, Israel 1998, France 2000, and Russia 1999) were associated with severe complications, including neuroinvasive disease [6-11]. In 1999, WNV was detected for the first time in the Western Hemisphere during the outbreak in New York [12]. In the past few years, in European countries, WNV neuroinvasive disease was notified every year: Italy (2008-2012), Hungary (2008-2012), Romania (2010-2012) and Greece (2010-2012). In July 2012, the first outbreak of WNV disease has occurred in Serbia with 44 laboratory diagnosed cases and six deaths [13].

We are presenting the first human cases of WNV infection in Croatia, August-September, 2012 and the results of seroepidemiological study performed after the transmission season 2012 in Osijek-Baranja County, a region with the highest incidence of WNV neuroinvasive infection during 2012.

MATERIALS AND METHODS

During September-November 2012, a total of 54 serum samples and 15 cerebrospinal fluid (CSF) samples from patients with clinical symptoms of WNV infection (neuroinvasive disease and WNV fever) were tested at the National Reference Laboratory for Arboviruses, Croatian National Institute of Public Health (CNIPH) for the presence of WNV IgM and IgG antibodies using commercial ELISA (Euroimmun, Lübeck, Germany). Samples were obtained from hospitalized and non-hospitalized patients with clinical diagnosis of meningoencephalitis (27/50.0%), encephalitis (12/22.2%), non-specific febrile disease (13/24.1%) and febrile disease with rash (2/3.7%). IgM/IgG reactive samples were further tested for IgG avidity (Euroimmun, Lübeck, Germany) to confirm acute/recent WNV infection. The interpretation of

AI results was determined as follows: AI <40% = low avidity antibodies indicating acute/recent WNV infection; AI 40-60% = moderate avidity; AI >60% = high avidity antibodies indicating past WNV infection. Any IgM or IgG positive samples were evaluated at the OIE Reference Laboratory for West Nile Disease, Istituto G. Caporale, Teramo, Italy by plaque-reduction neutralization (PRNT) and micro-neutralization test (MNT) for result confirmation. Nine (5 CSF and 4 serum) samples were tested for WNV RNA using real-time pan-flavivirus RT-PCR and qRT-PCR assays for detection of WNV lineage 1 and 2 [14,15].

Following the outbreak of WNV infection, from September 2012 to February 2013, a total of 178 serum samples were collected from healthy inhabitants of Osijek-Baranja County aged 7-86 years where the incidence of neuroinvasive cases was highest to evaluate the seroprevalence of WNV.

RESULTS

Human cases of WNV neuroinvasive infection

Between 6 and 21 September 2012, 7 human cases of WNV neuroinvasive infection have been diagnosed in three eastern Croatian counties (Brod-Posavina, Osijek-Baranja and Vukovar-Srijem County), four of which were reported in Osijek-Baranja County (*Figure 1*). Patient's demographic and clinical characteristics are presented in the *Table 1*. The first case resided in northern Serbian province of Vojvodina during the incubation period, while the other represents autochthonous WNV cases.

Laboratory results

WNV IgM antibodies were detected in all patients. In six patients, IgG antibodies of low avidity were found indicating acute WNV infection. Using PRNT and MNT, WNV neutralizing antibodies were confirmed in all samples (*Table 1*). For two patients, paired serum samples were available, which showed a four-fold increase of WNV-specific antibody titre. None of the tested CSF samples were WNV antibody positive. Real-time RT-PCR was negative in all tested samples.

WNV IgG seroprevalence results

Of 178 tested subjects in Osijek-Baranja County, four (2.2%) were reactive for WNV IgG antibodies by ELISA (28-200 RU/ml) while one participant (0.6%) showed equivocal result (20 RU/ml). Two seropositive subjects were residents of urban settings (Osijek region) and two lived in rural area (Semeljci, Strizivojna) (*Figure 1*).

Table 1. Demographic, clinical and laboratory data of 7 patients with West Nile virus neuroinvasive disease in Croatia, August-September, 2012.

Characteristic	Results
Age	
Median	62 years
Range	48 - 77 years
Sex	
Male	3 (57.1%)
Female	4 (42.9%)
Area of residence	
Rural	5 (71.4%)
Urban	2 (28.6%)
Clinical symptoms	
Meningoencephalitis	5 (71.4%)
Meningoencephalitis, AFPa	2 (28.6%)
History of hypertension	4 (57.1%)
Outcome	
Recovered	5 (71.4%)
Persistent neurologic sequelae	2 (28.6%)
Laboratory results	
WNV ELISA - IgM (Ratio)b	1.7 - 4.1
WNV ELISA - IgG (RU/ml)c	40 - 170
WNV IgG avidity	11-38%
WNV PRNTd (Titre)	5 - 40
WNV MNTe (Titre)	5 - 80

aAFP = acute flaccid paralysis; bRatio <0.8 neg, 0.8-1.1 equivocal, >1.1 pos; c<16 neg, 16-22 equivocal, >22 pos; dPRNT = plaque-reduction neutralization test; eMNT = micro-neutralization test

Figure 1. Map of Croatia representing human WNV neuroinvasive infection notified in 2012 and IgG positive participants.

DISCUSSION

In Croatia, clinical cases of human WNV infection were not reported so far although cases occurred in many European countries (Italy and Hungary 2008-2012, Spain 2009, Romania and Greece, 2010-2012) [13]. However, serologic evidence for the presence of WNV in Croatia dates back to 1970s. These studies were usually based on the detection of hemagglutination inhibiting (HI) antibodies that are group-specific. In two studies conducted among residents of the Island Brač in 1970 and 1974, the prevalence of HI antibodies was 4.9% and 0.28%, respectively [16]. In another survey (1980), positive HI results were found in 1.7% Croatian inhabitants: 1.2% from North-East Croatia, 3.4% from Middle Dalmatia and 0.8% from Southern Dalmatia [17]. In a study conducted in 2007 among voluntary blood donors in north-east Croatia, antibodies against WNV were detected in 0.3% participants by neu-

tralization [18]. A pilot study performed in a group of 306 randomly selected adult people aged 30-60 years in 2011 showed similar results. Only one participant (0.3%) from eastern Croatia (Osijek-Baranja County) showed positive neutralizing WNV antibodies (data from the CNIPH). In addition to humans, antibodies to WNV were detected in brown bears in 1993 [19] and horses in 2001-2002 [20]. WNV infection in horses was documented in a large study during 2011-2012, the results of which indicate a possible endemic appearance of WNV in Croatia (WNV activity was documented in at least 9 counties) [21].

The first clinical cases of WNV neuroinvasive disease in Croatia were laboratory confirmed in September 2012. A serological survey in sentinel horses demonstrated asymptomatic acute WNV infection (detection of IgM antibodies) in 12 animals in the same counties where human cases were reported (data from the Faculty of Veterinary Medicine University of Zagreb). The exception is Osijek-Baranja County where human clinical cases, but not infections in horses were reported. These results could be explained by searching the horses for WNV before the increase in vector activity. In addition, acutely infected horses were documented in Virovitica-Podravina County in which there were no human cases reported. Since most infected people do not show any symptoms or have non-specific febrile disease, it is possible that many WNV infections remained unrecognized.

In areas where cases of WNV infection occurred and in neighboring municipalities, mosquito control measures (adulticidal and larvicidal treatment) were immediately implemented.

Results of seroepidemiological study performed after the transmission season 2012 in Osijek-Baranja County showed that the seroprevalence rate in healthy inhabitants increased compared to 2011. The increase in WNV seropositivity in these two consecutive years indicates increased viral activity during season 2012. These results are in accordance with the first reported clinical cases.

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Sažetak

Infekcija ljudi virusom Zapadnog Nila u istočnoj Hrvatskoj, kolovoz – rujan 2012.

U razdoblju od 6. do 21. rujna 2012. zabilježeni su prvi humani klinički slučajevi neuroinvazivne infekcije virusom Zapadnog Nila (VZN) u tri hrvatske županije (Brodsko-posavska, Osječko-baranjska i Vukovarsko-srijemska). U prvom se slučaju vjerojatno radilo o infekciji podrijetlom iz Srbije, gdje je bolesnik boravio tijekom inkubacijskog perioda, a u ostalih bolesnika o autohtonoj infekciji VZN-om. Pet je bolesnika imalo kliničku sliku meningoencefalitisa, dok je u dva bolesnika nakon početnih simptoma meningoencefalitisa nastupila akutna mlohava kljenut. U svih su dokazana VZN IgG i/ili IgM protutijela pomoću imunoenzimnog testa (ELISA) te su potvrđena neutralizacijskim testom redukcije plakova (PRNT) i mikroneutralizacijskim testom (MNT). Po završetku sezone sakupljeno je ukupno 178 uzoraka seruma zdravih osoba s područja Osječko-baranjske županije, gdje je tijekom epidemije zabilježen najveći broj slučajeva. Pomoću ELISA testa VZN IgG protutijela dokazana su u 4 (2,2%) ispitanika. Ti rezultati ukazuju na povećanu cirkulaciju VZN-a na tom području u usporedbi sa sezonom 2011. godine, što je rezultiralo prvim humanim kliničkim slučajevima.

Ključne riječi: virus Zapadnog Nila; pojava; seroprevalencija; Hrvatska**.**

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CLINICAL ASPECTS OF WEST NILE VIRUS INFECTIONS IN HUMANS IN CROATIA

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Summary

During August and September of the year 2012th there were 5 patients with meningoencephalitis treated at Clinic of Infective Diseases, University Hospital Centre Osijek. In all of them West Nile virus was confirmed to be cause, serologically (ELISA IgG and IgM positive). These are the first confirmed cases of West Nile encephalitis in Eastern Slavonia, Croatia (Budimci, Vukovar, Belisće, Kuševac, Petrijevci). Patients ranged in age from 48 to 77 years. One year ago, three of these patients were treated in Neurology; two of them due to the stroke, and third because the vertigo. Patients were admited to the hospital on the 4th to 6th day from the begining of general infectious symptoms, and the first day of neurological symptoms. Two patients had confusion, disorientation and hallucinations, two patients had somnolence and sopor, and two patients had tremor. During first two days of treatment, all patients had raised body temperature in range from 37.2° C to 38.3° C. Cerebrospinal fluid analysis in all patients spoke in favor of serous meningitis. The number of leukocytes (mainly lymphocytes) ranged from 10 to 517 x10^6/L, there was a moderate protein elevation and marginal lactate elevation. Glucose and chlorides were within normal range. All patients had EEG changes dysrithmic, diffuse or laterally. CT scan of the brain was normal in two patients, two patients had diffuse brain atrophy, and one had chronic vascular lesions. MRI scan of the brain in two patients was normal. Patients were treated with anti-edematous therapy with infusion 10% Mannitol and 10% Dextrose and other symptomatic therapy. Clinical signs improved within 5 days of treatment. Hospitalization lasted 2-3 weeks. All patients were discharged without neurological sequelae.

Keywords: West Nile virus infection; meningoencephalitis; Eastern Slavonia; Croatia.

INTRODUCTION

About 80% of West Nile virus (WNV) infections are asymptomatic. Most WNV infections are mild and often clinically unapparent. Approximately 20% of those infected develop a generally mild illness (West Nile fever). The incubation period is thought to range from 3 to 14 days [1,2,3]. Symptoms generally last 3 to 6 days.

The mild form of WNV infection as a febrile illness of sudden onset often accompanied by: malaise, headache, anorexia, myalgia, nausea, rash, vomiting, lymphadenopathy, eye pain [4,5,6]. Symptoms occurring among patients hospitalized with severe disease include: fever, gastrointestinal symptoms, weakness, severe neurological disease. A minority of patients with severe disease developed a maculopapular or morbilliform rash involving the neck, trunk, arms, or legs [7]. Approximately 1 in 150 infections will result in severe neurological disease [7]. The most significant risk factor for developing severe neurological disease is advanced age [1].

Neurological presentations include: encephalitis, meningoencephalitis, meningitis, West Nile poliomyelitis, acute flaccid paralysis, ataxia, extrapyramidal signs, optic neuritis, cranial nerve abnormalities, polyradiculitis [4,5,8]. Encephalitis is more commonly reported than meningitis.

Signs and symptoms of neurological diseases include: high fever, severe headache, stiff neck, disorientation or confusion, sopor or coma, tremors or muscle jerking, lack of coordination, convulsions, pain, partial paralysis or sudden weakness [9,10]. Signs and symptoms of West Nile fever usually last a few days, but signs and symptoms of encephalitis or meningitis can linger for weeks, and certain neurological effects, such as muscle weakness, may be permanent.

Myocarditis, pancreatitis, and fulminant hepatitis have been rare described [11].

MATERIALS AND METHODS

Five patients with serologically confirmed West Nile virus infection and meningoencephalitis were analysed, according to following criteria shown in the table: age, sex, place of residence, personal anamnesis, tick bite, swimming in stagnant water, period of disease before hospitalization, highest body temperature, headache, vomiting, duration of neurological symptoms before hospitalization, photophobia, neck stiffnes, neurological symptoms, white blood cell count, platelet count, laboratory information on levels of alanine aminotransferase, aspartate aminotransferase, gamma-glutamyl transferase and urea, liquor white blood cell count, electroencephalography, CT and MRI scan of the brain, serological tests (ELISA - Enzyme-linked immunosorbent assay test).

RESULTS AND DISCUSSION

Data on our patients are shown in following six tables, sorted according to different criteria.

Table 1. Details from personal anamnesis in patients

Patient	Age (in years)	Sex	Place of residence	Personal anamnesis	Tick bite	Swimming in stagnant water
1. D.M.	48	F	Budimci	Vertigo	-	-
2. S.K.	77	F	Vukovar	Hypertension	-	-
3. P.B.	76	M	Belišće	Stroke, hypertension	-	-
4. R.V.	48	F	Kuševac	-	-	-
5. V.S.	65	M	Petrijevci	Hypertension, chr. radiculopathy, stroke	-	-

Table 2. General symptoms in patients

Patient	Period of disease before hospitalization (in days)	Highest body temperature	Headache	Vomiting
1. D.M.	4	38 ºC	+	+
2. S.K.	3	38,5 ºC	+	- (Nausea)
3. P.B.	3	38 ºC	+	- (Nausea)
4. R.V.	5	38,3 ºC	+	+
5. V.S.	6	38,5 ºC	+	+

Table 3. Neurological symptoms of patients

Patient	Duration of neurological symptoms before hospitalization (in days)	Photophobia	Neck stiffness	Neurological symptoms
1. D.M.	1.	+	+	Apathy, somnolence, sopor
2. S.K.	1.	+	+	Anxiety, irritability, hallucination
3. P.B.	1.	-	-	Anxiety, irritability hallucination, tremor
4. R.V.	1.	+	+	Apathy, somnolence
5. V.S.	1.	-	-	Tremor

Table 4. Blood analysis data

Patient	White blood cell count 3,4 – 9,7 x10^9/L	Platelet count 158 – 424x10^9/L	AST (11-38 U/l) ALT (12-48 U/l) GGT (11-55 U/l)	Urea 2,8-8,3 mmol/L
1. D.M.	5,0	214	AST 64, ALT 99, GGT 42	5,5
2. S.K.	11,0	190	AST 37, ALT 80, GGT 49	12
3. P.B.	5,1	143	Normal AST and ALT, GGT 94	13,2
4. R.V.	14,1	187	Normal value	6,2
5. V.S.	3,5	84	AST 51, ALT 65, GGT 41	5,8

Table 5. Diagnostic tests used

Patients/ Lumbar puncture done on day	Liquor white blood cell count $x10^6$/L	Electro-encephalography (EEG)	CT and MRI Scan of the brain
1. D.M. 14 .day	10 (ly 100%)	Diffuse dysrithmic	CT scan of the brain - normal
2. S.K. 1. day	517 (ly 15, mono 3, neutro 82 %)	Unilateral dysrithmic	CT scan of the brain - diffuse brain atrophy
3. P.B. 2. day	120 (ly 26, mono 9, neutro 65%)	Difusse dysrithmic	CT scan of the brain - diffuse brain atrophy MRI scan of the brain - Normal
4. R.V. 1. day	272 (ly 74, mono 19, neutro 16 %)	Unilateral dysrithmic	CT scan of the brain - normal
5. V.S. 12. day	17 (ly 75, mono 23, neutro 2%)	-	MR of the brain - chronic vascular lesion

Table 6. Serological test results

Patients	Day of disease when serum sample was taken	ELISA Positive >1,1 RU/mL IgM	ELISA Positive >22 RU/mL IgG
1. D.M.	21.	1,7	132
2. S.K.	15.	3,3	40
3. P.B.	6.	3,2	Neg
4. R.V.	13.	4,1	125
5. V.S.	8.	3,5	Neg

All patients were residents of different settlements from eastern Slavonia. Patients ranged in age from 48 to 77 years. Most of them had some chronic illness. Three of these patients were treated in Neurology; two of them due to the stroke, and third because the vertigo. During first two days of treatment, all patients had raised body temperature in range from 37.2º C to 38.3º C. Patients were admited to the hospital on the 4th to 6th day from the beginning of general infectious symptoms, and the first day of neurological symptoms. Two patients had confusion, disorientation and hallucinations, two patients had somnolence and sopor, and two patients had tremor. All of them had headache and most of them vomiting. Three patients had photophobia and neck stiffness. One had leukopenia, two thrombocytopenia and three elevated aminotransferase levels.

Cerebrospinal fluid analysis in all patients spoke in favor of serous meningitis. The number of leukocytes (mainly lymphocytes) ranged from 10 to 517 x10^6/L. All patients had EEG changes dysrithmic, diffuse or laterally. CT scan of the brain was normal in two patients, two patients had diffuse brain atrophy, and one had chronic vascular lesions. MRI scan of the brain in two patients was normal. Serum samples were collected 6th to 21th day of illnes, and in all patients West Nile virus was proved to be cause, serologically (ELISA IgG and IgM positive).

CONCLUSION

During August and September of the year 2012th there were 5 patients with meningoencephalitis treated at Clinic of Infective Diseases, Osijek University Hospital Centre. In all of them West Nile virus was confirmed to be cause, serologically (ELISA IgG and IgM positive). These are the first confirmed cases of West Nile encephalitis in Eastern Slavonia, Croatia (Budimci, Vukovar, Belisce, Kusevac, Petrijevci).

We showed clinical manifestations, neurological symptoms and certain laboratory tests in first patients with West Nile virus meningoencephalitis in Eastern Slavonia, Croatia.

Patients were treated with anti-edematous therapy with infusion 10% Mannitol and 10% Dextrose and other symptomatic therapy. Clinical signs improved within 5 days of treatment. Hospitalization lasted 2-3 weeks. All patients were discharged without neurological sequelae.

From now on, in differential diagnosis of patients with acute meningoencephalitis occuring during warmer months, West Nile virus infection must be taken in consideration.

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Sažetak

Klinička obilježja infekcije virusom Zapadnog Nila kod ljudi u Hrvatskoj

Tijekom kolovoza i rujna 2012. godine u Klinici za infektologiju KBC-a Osijek liječeno je pet bolesnika s meningoencefalitisom, u kojih je serološki (ELISA IgG i IgM pozitivni) dokazan virus Zapadnog Nila. To su prvi dokazani slučajevi meningoencefalitisa uzrokovanog virusom Zapadnog Nila u istočnoj Slavoniji (Budimci, Vukovar, Belišće, Kuševac, Petrijevci). Bolesnici su bili u dobi od 48 do 77 godina. Tri su se bolesnika godinu prije liječila na neurologiji: dva zbog cerebrovaskularnog inzulta, a treći zbog vertiginoznog sindroma. Hospitalizirani su od četvrtog do šestog dana prisutnih općih infektivnih simptoma, a prvog dana prisutnog poremećaja svijesti. Smetenost, dezorijentiranost i halucinacije imala su dva bolesnika. Somnolencija i sopor bili su prisutni također kod dva bolesnika, a kod dva bolesnika bio je prisutan i tremor.

Povišenu temperaturu svi su bolesnici imali prva dva dana liječenja i ona je iznosila od 37,2 °C do 38,3 °C. U svih je bolesnika nalaz likvora govorio u prilog seroznog meningitisa. Broj leukocita (pretežno limfocita) varirao je od 10 do 517 x10^6/L. Prisutna je bila umjerena proteinorahija i granično povišene vrijednosti laktata. Glukoza i kloridi bili su u granicama normale. U svih bolesnika EEG je bio dizritmički promijenjen, difuzno ili postranično. CT mozga bio je uredan kod dva bolesnika, kod dva bolesnika bila je prisutna difuzna atrofija mozga, a kod jednog kronična vaskularna lezija. Nalaz MR-a mozga kod dva bolesnika bio je uredan. Uz antiedematoznu terapiju (infuzije 10% manitola i 10% dekstroze) i drugu simptomatsku terapiju, unutar pet dana liječenja uslijedilo je poboljšanje. Bolničko liječenje trajalo je 2 – 3 tjedna. Svi su otpušteni bez neuroloških posljedica.

Ključne riječi: infekcija virusom Zapadnog Nila; meningoencefalitis; istočna Slavonija; Hrvatska.

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WEST NILE VIRUS MONITORING IN WILD BIRDS IN SLOVENIA

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Summary

Wild bird carcasses were collected through passive monitoring of wild bird mortality. In necropsy, brain samples were taken from dead wild birds in 2010, 2011 and 2012. Samples were used for detection of West Nile virus by reverse transcriptase-polymerase chain reaction (RT-PCR). No WNV nucleic acid was detected by RT-PCR. In the light of the data presented there is no evidence of wild bird mortality in Slovenia due to WNV activity.

Keywords: West Nile virus; wild birds; Slovenia.

INTRODUCTION

The presence of West Nile virus (WNV) in Europe has been known for decades. Virus transmission is related to the ornithophilic mosquitos. Wild birds are an important natural amplifying host for the virus. Humans, horses, and some other mammals are considered dead-end hosts [1]. Cases of the West Nile disease (WND) in humans and horses were reported in Europe and from Mediterranean basin [1,2,3]. In Europe, wild bird mortality related to WNV infection was only seen very seldom [1,3]. Recently, WNV was detected in dead wild birds in Hungary in 2004 [4]. Since then the spread of the virus in wild birds was observed in Hungary, Austria [5], Italy [6] and Greece [7]. In Slovenia, the presence of WNV was reported on the basis of serological studies on forest workers [8], horses [9] and wild birds [10]. Birds testing positive were found among passerines and free-living domestic pigeons [10,11,12].

The main goal of our study was to obtain further information about the role of wild birds in the ecology of WNV in Slovenia.

MATERIALS AND METHODS

Samples

From 2010 to the end of 2012, wild birds of various orders were collected as part of the passive surveillance program for detection of avian influenza viruses. Birds were dissected, and brain specimens stored at -70 Cº until nucleic acid extraction was performed.

Molecular analysis

Viral RNA was extracted from the brain homogenates by using QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) with an input volume of 140 µl and elution volume of 60 µl, in accordance with the manufacturer's instructions.

For detection of the viral RNA, reverse transcriptase-polymerase chain reaction (RT-PCR), based in the conserved non-structural protein 5 (NS5) was used for its ability to detect all members of the Japanese encephalitis virus antigenic group of flaviviruses [13]. RT-PCR was performed with Qiagen One-Step RT-PCR kit by using 2 µl RNA and 16pmol of forward primer: 5`-GARTGGATGACVACRGAAGACATGCT-3` and reverse primer: 5`-GGGGTCTCCTCTAACCTCTAGTCCTT-3` in a 20 µl total reaction volume.

The RT-PCR products were analysed by electrophoresis in a 1.8% agarose gel stained with ethidium bromide.

RESULTS

In behalf of passive monitoring of wild bird mortality in 2010, 2011 and 2012 a total of 58 brain tissue samples were taken during a routine necropsy from 15 different species of wild birds (*Table 1*). All samples were screened for the WNV nucleic acid using RT-PCR. All 58 samples tested for the presence of the West Nile viral nucleic acid by RT-PCR were negative.

Table 1. The number of wild birds sampled and tested

Species of wild birds		Number of wild birds sampled/ tested		
		2011	2012	2013
Common Buzzard	*Buteo buteo*	6	3	1
Mallard	*Anas platyrhynchos*	1	3	2
Mute Swan	*Cygnos olor*	7	1	10
White Stork	*Ciconia ciconia*	5	1	-
European Sparowhawk	*Accipiter nisus*	1	-	-
Common Pigeon	*Columba livia*	1	2	-
Common Kestrel	*Falco tinnunculus*	2	-	-
Barn Swallow	*Hirundo rustica*	1	-	-
Blackbird	*Turdus merula*	-	1	3
Gull	*Larus* spp.	-	-	1
Grey Heron	*Ardea cinerea*			1
Hooded Crow	*Corvus corone cornix*	-	-	2
Hawfinch	*Coccothraustes coccothraustes*	-	-	1
Greenfinch	*Carduelis chloris*	-	1	-
Common Starling	*Sturnus vulgaris*	-	-	1
Total	**15 species**	**24**	**12**	**22**

DISCUSSION

In our study, all 58 wild birds tested negative by RT-PCR for the presence of the WNV nucleic acid. There was no evidence of wild bird mortality due to WNV activity in Slovenia. To our knowledge, WNV infections in free-living birds in Slovenia have never been associated with clinical symptoms or mortality (personal communication), although outbreaks and sporadic cases of the West Nile disease in wild birds were reported from the neighbouring countries [4,5,14]. In Europe, a fatal WNV infection in wild birds was first observed in Hungary in 2004, in a Goshawk (*Accipiter gentilis*) fledgling [4]. The isolated virus belonged to the lineage 2 WNV and was seen for the first time in Europe. Since then, passive monitoring of wild bird mortality in Hungary and Austria in 2008 and 2009 revealed that Goshawks, Sparrow hawks (*Accipiter nisus*), and other birds of prey were most commonly affected [5]. In Italy, a WNV strain belonging to lineage 2 was for the first time detected in the tissues of a Wild Collared Dove (*Streptopelia decaocto*) found dead in 2012 [6]. Moreover, WNV lineage 1 tested positive in European Magpie (*Pica pica*) and Eurasian Jay (*Garrulus glandarius*) during active surveillance in the same country in 2010

[14]. The reason of the explosive spread of the lineage 2 virus in Central Europe remains yet unclear [5]. It is possible that the virus was introduced to Hungary by the migratory birds [1,4,5]. The vertebrate reservoir host (e.g. free-living pigeons) is hypothesized for being responsible for the dissemination of the virus. In Hungary, specific antibodies were found in 70 % of pigeons [5]. In Italy, a WNV lineage 1 was isolated from pools of brain, kidneys, heart and spleen of one pigeon [15]. Among the resident species Magpies, Carrion Crows and pigeons are the most probable species involved in the endemic cycle of WNV [3]. In our country, the prevalence of antibodies against WNV in free-living Domestic Pigeons (*Columba livia*) was 12.4 % (23/186). Pigeons were caught and sampled at different locations in the city of Ljubljana, the capital of Slovenia [11]. Pigeons seem to be particularly suitable reservoirs and the infection of urban pigeons might increase the risk of human infections [5].

Considering reports of the increased WNV activity in some European countries further systematic research is needed to understand the epidemiology end the ecology of WNV in Slovenia.

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Sažetak

Praćenje virusa Zapadnog Nila u divljih ptica u Sloveniji

Lešine divljih ptica sakupljene su zbog praćenja mortaliteta. Uzorci mozga uginulih ptica bili su uzimani pri razudbi tijekom 2010., 2011. i 2012. godine te su bili pretraženi na virus Zapadnog Nila lančanom reakcijom polimerazom uz prethodnu reverznu transkripciju (RT-PCR). Nukleinska kiselina za virus Zapadnog Nila nije bila dokazana RT-PCR-om. Na osnovi tih rezultata može se ustvrditi da aktivnost virusa Zapadnog Nila nije dokazana u divljih ptica u Sloveniji.

Ključne riječi: virus Zapadnog Nila; divlje ptice; Slovenija.

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WEST NILE VIRUS SEROSURVEILLANCE IN HORSES IN CROATIA DURING THE 2012 TRANSMISSION SEASON

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Summary

In 2012 the active WNV surveillance system in horses was introduced in Croatia. Between June and October 2012, in six Croatian counties, 1804 horse sera were sampled and tested for IgM WNV antibodies as the confirmation of an acute infection. Additional 1472 samples were tested for the presence of IgG WNV antibodies in the seroprevalence study. The study showed the increased seroprevalence of WNV infection in horses and acute infections in horses in three Eastern Croatian counties. In the same transmission season, the first human WNV clinical cases were reported in the counties with a confirmed increase in WNV seroprevalence in horses. Even more, the first detection of horses acutely infected with WNV had been two weeks before the first confirmed human clinical case. The results confirmed the active serosurveillance system in horses as a valuable tool for WNV surveillance in Croatia and as the source of important veterinary medicine and public health data.

Keywords: West Nile virus; serosurveillance; horses; public health; Croatia.

INTRODUCTION

West Nile virus (WNV) is a zoonotic mosquito-transmitted arbovirus belonging to the genus *Flavivirus* in the family *Flaviviridae*. The first isolation of West Nile virus was documented in Omogo in the West Nile District of Uganda from an adult woman [1]. Subsequently, the virus has been described to be widely distributed in many parts the Old World, such as Africa, the Middle East, Asia, Europe, and elsewhere [2]. During the summer of 1999, WNV was first detected in the Western

Hemisphere in the north-eastern United States of America [3]. For the last two decades the West Nile virus (WNV) infection associated disease outbreaks are occurring worldwide, making it the pathogen of major global public health concern. In Europe in the last few transmission seasons an increased number of WNV infections has been noted in humans and animals and a concern has been raised regarding public and animal health.

In WNV natural cycle birds normally act as amplifying hosts whereas mosquitoes, mainly of the genera *Culex*, *Aedes* and *Ochlerotatus*, play the vector role. In this cycle humans, horses and other mammals are regarded as incidental or dead-end hosts. The members of the order *Passeriformes* (jays, blackbirds, finches, sparrows, crows) and *Columbiformes* (collared doves) seem to be important in maintaining the virus in nature because of their high viraemias and, in opposite, because of their presumed low and transient viraemias, humans and horses are not considered important in the natural transmission cycle [4].

Several WNV lineages have been described so far [5]. In Europe, the lineage 1 is widespread and further segregates into different subclades. Two main routes of lineage 1 dispersion have been identified in Europe, one in Eastern and the second one in Western Europe [6]. Lineage 2 is mainly present in sub-Saharan Africa and Madagascar, but in the last decade was also identified in the Eastern Europe, first time detected in Hungary in 2004 [7]. Since then, lineage 2 has been detected in Rumania [8], Greece [9], Italy [10] Russia [11] and Austria [12]. Along with lineages 1 and 2, WNV lineage 3 has been isolated in the Rabensburg region of the Czech Republic [13] and lineage 4 has been identified in the Southern Russia [14]. WNV strains differ considerably in virulence and neuroinvasiveness. Since neuroinvasive isolates mainly belong to lineage 1, lineage 2 strains are considered to be less virulent. Recent data, however, indicate that the several highly virulent and neuroinvasive strains of lineage 2 WNV have been detected in Southern Africa [15].

The fact that West Nile virus is widespread in many European countries and outbreaks in humans with fatal end cases as the evidence of virus endemisation [16] highlighted the necessity of global surveillance system introduction.

Different WNV surveillance systems have been established in different countries in accordance with the epidemiological situations. In general, WNV control systems include surveillance in human and animal populations and entomological surveillance. All surveillance systems can be performed as passive and/or active systems.

The aim of the human passive surveillance system is the detection of infection in humans and the estimation of its diffusion through the systematic analysis of newly emerging clinical cases. The active human surveillance includes the serolo-

gical testing of people who live or work in the areas of documented WNV circulation. In general, the results of human surveillance systems, especially passive, are inadequate because the results are usually belated in order to introduce specific prophylactic measures.

The passive surveillance of the clinical cases in animal populations gives important veterinary medicine and public health data. During the first three seasons of WNV outbreaks in North America avian mortality proved to be extensive. Natural fatal infections have been confirmed based on the positive laboratory tests on over 28,000 carcasses of 198 bird species [17]. The surveillance system based on the monitoring of bird mortality has been proven as a valuable tool for assessing the risk of West Nile virus infection in humans and even in equines in North America [18]. On the contrary, during many WNV outbreaks in equine and human populations in Europe, an increased mortality in birds was not reported, so the passive surveillance in birds could only be a part of a part of more complex WNV surveillance system in Europe. Another important limiting factor of dead bird surveillance are the results that are clearly depending on carcasses submission by citizens and the public awareness of WNV importance.

The passive surveillance of horses appeared to be the most cost-effective system in the current European context [19], but it depends on the number and the presence of specialized veterinary clinics. That is the reason that in some countries and regions equine WNV clinical cases can go unrecognized. For these countries, like Croatia is, with very few specialized equine clinics, the passive surveillance of horses is inappropriate and the active surveillance apparently represents the best WNV surveillance system.

Due to mostly unspecific clinical signs even the neurological form of WNV infection in horses, could be misdiagnosed as some other disease without laboratory confirmation. There is even some possibility that Mraclin Disease described in Croatia in 1938, according to clinical signs was unrecognised outbreak of WNV infection in horses [20].

In Croatia, during the years 2010 and 2011 [21], the advantage of the active horse surveillance over the passive one was confirmed when WNV activity was observed in Eastern Croatia without any reported horse clinical case. Even more, in 2012 the first human WNV clinical cases occurred in the region where the highest seroprevalence in horses had been recorded in the previous two years, highlighting the importance of the active horse surveillance for public health data.

On the basis of the 2010 and 2011 surveillance results, in 2012 active WNV surveillance in the sentinel horses in Croatia was conducted in six counties with the highest WNV seroprevalence in horses confirmed in the previous two years.

MATERIALS AND METHODS

Between June and October 2012, 1804 horse sera were sampled for IgM WNV antibodies testing as the confirmation of an acute infection. Additionally, 1472 sera samples were tested for the presence of IgG WNV antibodies in the seroprevalence study. The animals, which were randomly selected, originated from six Croatian counties, namely: County of Brod-Posavina, County of Istra, County of Osijek-Baranja, County of Požega-Slavonia, County of Virovitica-Podravina and County of Vukovar-Srijem with the confirmed high WNV seroprevalence in horses in the previous two years.

The criterion for the selection of animals was that they were not moved in the international transport nor moved between counties. The age of animals was from six months to more than 25 years. The horses included in this study had no WNV vaccination history and at the time of sampling all animals were asymptomatic.

The horse serum samples were tested for WNV IgM antibodies using a commercial enzyme-linked immunosorbent assay (ID Screen West Nile IgM Capture, ID.VET, Montpellier, France) and for WNV IgG antibodies using a commercial competitive enzyme-linked immunosorbent assay c-ELISA (ID screen West Nile competition ELISA kit, ID.VET, Montpellier, France) in accordance with the manufacturer's instructions.

RESULTS

Study of acute WNV infection in horses

Out of 1084 horse sera samples tested for IgM WNV antibodies, 12 were positive (0,7%). Positive sera samples originated from the three counties. Six IgM positive samples were taken from horses on five different locations of County of Vukovar-Srijem from July 20th till August 1st. The seroprevalence of acute WNV infection in this county was 1,3%. The same seroprevalence of acutely infected horses was found in Virovitica-Podravina County with the confirmation of four acutely infected horses on three different locations. The horse sera were sampled during September 2012. Two more acute infections were confirmed in Brod-Posavina County, on different locations, with overall seroprevalence on county level of 0.9%. The sera were sampled during September 2012. In other three counties, Osijek-Baranja, Istra, Požega-Slavonia, acutely infected horses were not found. Horse sera from these counties were sampled from 15th June to 15th July in Osijek-Baranja County and during September 2012 in Istra and Požega-Slavonia counties (*Table 1, Figure 1*).

Table 1. The seroprevalence of IgG and IgM WNV antibodies in six Croatian counties included in WNV active horse serosurveillance during 2012

COUNTY	IgG			IgM		
	Number of samples	Number of positive samples	Seropre-valence (%)	Number of samples	Number of positive samples	Seropre-valence (%)
Brod-Posavina	184	32	**17,4**	216	2	**0,9**
Istra	276	5	**1,8**	177	0	**0,0**
Osijek-Baranja	257	26	**10,1**	360	0	**0,0**
Požega-Slavonia	276	6	**2,2**	283	0	**0,0**
Virovitica-Podravina	276	36	**13,0**	313	4	**1,3**
Vukovar-Srijem	203	23	**11,3**	455	6	**1,3**
Total	**1472**	**128**	**8,7**	**1804**	**12**	**0,7**

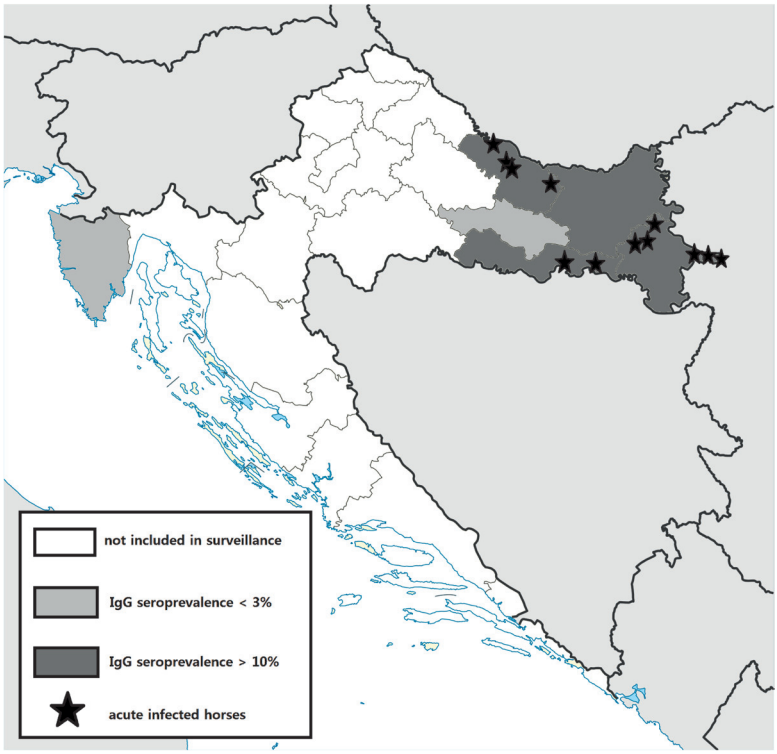

Figure 1. The seroprevalence of IgG WNV antibodies on county level and acutely infected horses during the transmission season 2012

WNV seroprevalence study in horses

In total, 1472 horse sera samples were tested for IgG WNV antibodies in the seroprevalence study for the transmission season 2012. The seroprevalences varied considerably between counties and the average seroprevalence was 8,7 %. The highest seroprevalence was found in County of Brod-Posavina with 32 out of 184 IgG positive samples (17,4%). Seroprevalence over 10% was found in three more counties. In County of Virovitica-Podravina 36 out of 276 horse sera samples were positive (13,0%), in County of Vukovar-Srijem 23 out of 203 tested samples (11,3%) were positive and finally, out of 257 horse sera samples 26 were positive in County of Osijek-Baranja (10,1%). Much lower seroprevalences were recorded in other two counties. County of Požega-Slavonia had only six out of 276 samples positive (2,2%) and Istra County five out of 276 (1,8%) (*Table 1, Figure 1*).

DISCUSSION

In Croatia, WNV serologically positive horses were detected more than 10 years ago [22] and active surveillance in horses was established in 2010. During the 2010 and 2011 the highest seroprevalence was observed in the same three counties in Eastern Croatia (County of Požega-Slavonia, County of Osijek-Baranja and County of Vukovar-Srijem) without significant differences in the seroprevalences on county level in these two consecutive seasons [21]. In this study we present high seroprevalences in sentinel horses, of more than 10%, in four out of six counties included in WNV surveillance during 2012. Like in the previous two years, the high seroprevalences were recorded in County of Osijek-Baranja, and in County of Vukovar-Srijem, but with an increase in 2012. Furthermore, high seroprevalence levels in the year 2012 were found in County of Brod-Posavina and County of Virovitica-Podravina with a drastic increase comparing to 2010 and 2011. On the contrary, the seroprevalence in Požega-Slavonia County was decreased in comparison with previous seasons, as well as the seroprevalence in County of Istra.. During 2012, the first evidence of WNV neuroinvasive clinical cases in humans were reported in Osijek-Baranja, Vukovar-Srijem and Brod-Posavina counties [23]. Many authors' studies noticed a correlation between WNV activity in animal population and the prediction of WNV human clinical cases occurrence [24,25,26]. The results of our study confirmed this correlation with the evidence of human neuroinvasive clinical cases in counties with high WNV seroprevalence in horses that is increasing.

Acute infections in horses were confirmed, for the first time in Croatia, during the surveillance in the transmission season 2012. Acute infection took places in the three counties with the increase of WNV seroprevalence. As referred in the outbre-

aks in other countries, acute infections in equids usually occurred for some time before the detection of WNV cases in humans [27,28]. The confirmation of acutely infected horses in County of Vukovar-Srijem, more than 2 weeks before the first human clinical cases occurred, highlights the importance of active horse surveillance as a predictive tool in public health in Croatia.

Acutely infected horses were also found in County of Brod-Posavina during the testing of horse sera sampled after the reporting of first human clinical cases. These results confirmed a high viral activity in this region in the transmission season 2012. In County of Virovitica-Podravina acutely infected horses were also confirmed on three distinct locations but with no evidence of human clinical cases. Lack of human cases could easily be the result of not reporting and of insufficient awareness of physicians because this county was not considered as the region of high risk. On the contrary, County of Osijek-Baranja had four human clinical cases with no confirmed acute infections in horses. It should be kept in mind that the time of sera sampling in this county had been before the peak season of vector activity so the the possibility of acute infections in horses had been very low.

The absence of reported equine clinical cases during the transmission season 2012, despite high seroprevalence, confirmed acute infection in horses and human clinical cases could be the result of the different virulence of Croatian WNV field strains for horses and humans. It is more likely that the absence of reported equine clinical cases is the result of not reporting or not recognizing WNV infection related symptoms in horses. Anyway, the absence of reported clinical cases emphasises the deficiencies of WNV passive surveillance in horses in Croatia.

In conclusion, the results of the active serosurveillance in the horse population in Croatia during the year 2012 showed the increase of WNV activity in the eastern part of Croatia, the same area where the viral activity had been detected in the previous two years. The increase in seroprevalence and, for the first time, the evidence of acute infections in horses was observed in the same region where human WNV clinical cases were reported afterwards. The results confirmed the active serosurveillance system in horses as the most suitable way of WNV surveillance in Croatia. Collecting data through the active serosurveillance in horses represents an excellent WNV early warning system and could be used as a guideline for the establishment of adequate control measures in public health.

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Sažetak

Serološko istraživanje infekcije virusom Zapadnog Nila u konja u Hrvatskoj 2012. godine

Tijekom 2012. godine praćenje proširenosti infekcije virusom Zapadnog Nila u Republici Hrvatskoj provođeno je kontrolom proširenosti infekcije u asimptomatskih konja. Tijekom sezone prijenosa, od lipnja do listopada 2012. godine, pretražena su 1.804 uzorka seruma konja na prisutnost IgM specifičnih protutijela u svrhu dokazivanja akutnih infekcija virusom Zapadnog Nila. Povrh toga, radi određivanja seroprevalencije, pretražena su 1.472 uzorka seruma konja na prisutnost IgG protutijela. Programom je bilo obuhvaćeno šest županija Republike Hrvatske. Rezultati istraživanja pokazali su porast seroprevalencije u četiri županije istočne Hrvatske, dok su akutno zaražene životinje dokazane u tri županije istog područja. Tijekom istog razdoblja zabilježeni su prvi klinički slučajevi bolesti Zapadnog Nila u ljudi na području županija s dokazanim porastom seroprevalencije u konja. Akutne infekcije konja dokazane su dva tjedna prije prvih kliničkih slučajeva u ljudi. Prikazani rezultati potvrđuju da je provođenje aktivne kontrole konja na prisutnost infekcije virusom Zapadnog Nila iznimno važno za kontrolu te bolesti u Republici Hrvatskoj, kako za veterinarsku medicinu, tako i za javno zdravstvo.

Ključne riječi: virus Zapadnog Nila; seroprevalencija; konji; javno zdravstvo.

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CURRENT EPIZOOTIOLOGICAL SITUATION REGARDING THE WEST NILE VIRUS IN HORSES IN SERBIA

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Summary

The West Nile virus (WNV) was isolated in Uganda in 1937. Today, it is the most widely spread Flavivirus around the world. The transmission cycle of virus involves mosquitoes and birds, but humans and horses are accidental dead-end hosts. The infection in humans and horses is often asymptomatic, but the clinical signs of the disease include fever and meningitis and/or encephalitis.

Since the risk of spreading WNV infection is present and has become the threat for animal and human health, in 2010, for the first time in Serbia, we conducted a serological survey for the presence of anti-WNV antibodies in healthy horses. In total, 349 equine serum samples were randomly collected during 2010 from 28 locations in the Vojvodina province, Belgrade and Šabac. Samples were tested by homemade validated ELISA and by plaque reduction neutralization test (PRNT). The results showed that 12 % (42/349) of tested samples were found to be positive for anti-WNV IgG neutralizing antibodies.

In 2011, another study was carried out, with 255 tested stored equine serum samples. Among them, 28.6 % (72/255) were found positive. Commercial and in-house ELISA tested the presence of specific anti-WNV antibodies. Selected positive samples were additionally examined by confirmative PRNT.

With the aim of estimating the presence and circulation of WNV in horses after the outbreak of the disease in humans in Serbia in 2012, 130 equine serum samples were collected from six stables and one settlement in the Vojvodina province. Commercial ELISA and confirmative VNT test tested sera samples. Out of 130 serum samples tested, 64 (49.23 %) were found to be positive for WNV.

The results of our serosurvey indicate that WNV is circulating in the horse population in Serbia. During the summer 2012, the first cases of human WNV infection with fatal outco-

me in Serbia have been reported, followed with new cases and deaths in 2013. Although so far no cases of clinical disease in horses have been reported, the epidemiological situation in the neighbouring countries over the past few years warns us that it is necessary to conduct an extensive investigation of WNV infection in Serbia and to implement a special programme of surveillance and disease control.

Keywords: West Nile virus; horses; serology.

INTRODUCTION

The West Nile virus (WNV) is the most worldwide spread flavivirus. The virus is transmitted by mosquitoes and birds, which are reservoirs of infection. Humans and horses are also susceptible to infection, but as incidental dead-end hosts. Clinical manifestation of the disease with high morbidity and mortality generally occurs in birds and, to a lower extent, in humans and horses [1].

WNV belongs to the family *Flaviviridae,* genus *Flavivirus*, together with Japanese encephalitis virus, Murray Valley encephalitis, St. Louis encephalitis, Wesselsbron, Dengue and other viruses. The virus possesses single-stranded RNA and the virions are spherical, 50 nm in diameter. Phylogenetic analysis discovered 5 lineages of WNV. Lineage 1 has been detected on all continents, while lineage 2 was mostly limited to Sub-Saharan Africa. Recently, lineage 2 was introduced into Europe and explosively spread in its southern part. Although human cases of infection are usually asymptomatic, approximately 1 % of infected persons progress to the neuroinvasive form of infection. Horses rarely develop clinical signs of infection, yet 10 % of the attacked horses develop neurological disorder with the mortality rate of up to 50 % [2-5].

Spreading of the West Nile virus

WNV was first isolated from a febrile woman in Uganda in 1937 [6]. Since then, the virus had spread by migratory birds from Africa to Europe, Asia and North America. The virus was responsible for occasional outbreaks of the disease in Europe, the Middle East and Africa. Since the 1990s, however, the number of serious neuroinvasive cases in humans and horses has increased. In New York City, in North America, WNV infected 2.6 % of citizens and caused over 1,000 deaths and over 12,000 cases of meningitis and meningoencephalitis [7]. Human WNV disease was reported in: Morocco in 1996, Tunisia in 1997, the Czech Republic in 1997, Russia in 1999, France in 2003. Along with the human cases, enzootic infections in horses were reported in many countries: Italy, France, Croatia, Israel, Canada, Cuba, etc. In the last few years, WNV infections were reported mainly in the Mediterranean co-

untries and Eastern Europe [2]. In 2008 and 2009, WNV was diagnosed in Hungary and Austria in dead wild birds, in horses and humans [8, 9]. The new outbreaks with human fatality were reported in Greece in 2010 (http://:www.ecdc.europa.eu). In Italy in 2008, infection was registered in 251 horse stables with 794 cases and 5 deaths [10]. In Italy, the first human WNV infection with neuroinvasive clinical signs was reported in 2012 [4]. In Spain in 2010, the first outbreak of WNV infection was reported in horses, with 102 clinically ill horses and 15 deaths [10]. In Croatia, neuroinvasive WNV human cases were reported in 2012. Serological study conducted in horses in 2010/2011 in Croatia revealed the seroprevalence of 3.43 %, with the highest seroprevalence in Eastern Croatia near the Serbian state border [11].

Only a few investigations of WNV activity were conduced in the territory of former Yugoslavia. In one of them, authors described 0.5–7.9 % of seroprevalence among inhabitants tested between 1970 and the 1980s [12]. In 1972, WNV infection was serologically detected in 2.6–4.7 % of humans in Serbia [13]. Serological study in Croatia that was conducted in European brown bear revealed 4 seropositive animals out of 15 tested [14]. When WNV was re-introduced into Europe, the investigation of WNV activity in horses in Serbia was carried out for the first time in 2010, followed by new researches in 2011 and 2013.

WNV in horses in Serbia

Serum samples from 349 healthy horses were collected during 2009 and 2010 from the Belgrade district, the municipality of Šabac and 26 municipalities in Vojvodina (northern part of the country). Almost half of the total number of animals (48.4 %) were racing horses; 36 % of them were Lipizzans; 10.2 % ponies; 1.7 % Arabian horses; while 3.8 % of mixed breed. Samples were tested for the presence of specific anti-WNV antibodies by validated home-made ELISA, based on the use of recombinant envelope E (rE) protein [15] and plaque–reduction neutralization test (PRNT). Our results showed that 42/349 (12 %) of horses were IgG seropostive. Seropositive horses were detected in 14 out of 28 studied municipalities. 59.3 % thereof were mares and 40.7 % stallions (*Figure 1*). The animals showed neither neurological disorders nor signs of the disease. All 42 samples neutralized WNV; in one serum sample, high neutralization anti-Usutu antibodies were detected. This was the first time that antibodies against Usutu virus were detected in horses in Serbia. Average PRNT and ELISA titters were 140 and 10.4 respectively. None of the 30 randomly selected IgG negative sera tested were positive. The results of virus isolation on cell culture and real-time RT-PCR of the analysed sera were also negative.

Figure 1. WNV activity in Serbia. The left panel shows recently reported WNV activity in Europe and the Mediterranean basin in humans (empty squares) and horses (black circles). The right panel displays the municipalities (in grey) from Serbia's Vojvodina province, the Šabac municipality and the Belgrade district area sampled here, including those where WNV IgG positive horses were detected (in black).

A further serosurvey was conducted during the year 2011, when 255 archived horse serum samples were tested for the presence of WNV antibodies. Samples were collected from 7 different stables in the Vojvodina region and the Belgrade area 2007–2011. Horses included in this study were of different breeds and different ages. None of the tested horses showed clinical signs of the WNV disease.

Sera were tested for the presence of specific WNV antibodies by immunoassay blockage commercial ELISA kit (Ingezim West Nile Compac, Ingenasa, Spain) and in-house ELISA [15]. Out of 255 samples, 72 (28.6 %) resulted as seropositive (*Table 1*). The results gained by in-house ELISA and commercial ELISA matched in 90 % of the samples. Due to the cross reactivity between WNV and other viruses of Japanese encephalitis sero-complex, the selected seropositive samples were additionally examined by confirmative PRNT. Some of the samples were collected in very small quantities, so the PRNT test was performed on 48 samples that were positive in commercial ELISA. The presence of WNV antibodies using PRNT was confirmed in all 48 samples. The highest percentage of seropositive samples was detected within the horse population in Vršac, while the lowest seroprevalences to WNV was found in Surduk and Karadjordjevo regions (*Table 1*).

Table 1. The distribution of seroprevalence among horses in stables.

Location	No. of tested horses	Positive horses	%
Bečej	32	7	21.9
Kelebija	36	12	33.3
Karadjordjevo	21	3	14.3
Surduk	30	4	13.3
Pančevo	45	16	35.5
Vršac	45	18	40.0
Belgrade	43	12	27.9
Σ	**252**	**72**	**28.6**

Additionally, in order to estimate the circulation of WNV in horses after the outbreak of the disease in humans in Serbia in 2012, 130 equine serum samples were collected from November to December 2012. The horses originated from the Vojvodina Province, from 6 big stables (119 samples) and from 11 horses by different owners from the city of Novi Bečej. Among these samples, 30 sera were previously assayed. All tested horses were of different ages and different breeds, with no evidence of clinical signs of the disease and in good condition.

Serum samples from horses were tested for the presence of WNV IgG antibodies by a commercial ELISA (Ingezim West Nile Compac, Ingenasa, Spain), following the manufacturer's instructions. As the confirmation of the positive results, all blood sera samples that were positive by ELISA were tested for WNV specific neutralizing antibodies by virus neutralization test (VNT). VNT was conducted under bio safety level 3 conditions on Vero cells using twofold serial sera dilutions with the WNV NY-99 strain. Out of 130 serum samples tested, 64 (49.23 %) were found to be positive for WNV. The percentage of seropositive horses varied from 35 % to 64 %. The higher seroprevalence, with over 50 % of positive animals, were registered in central and western Bačka areas and the south part of the Banat area, along the rivers Tisza and Danube. Among 21 horses previously tested seronegative during 2009/2010, 8 seroconverted and tested positive in this study (*Table 2*).

Table 2. Presence of WNV IgG antibodies in horses per stable and location.

No.	Horse stables / locations	No. of tested horses	Results of anti-WNV IgG antibody ELISA test			Previously examined horses	Sero-conversion
			Positive	% posit.	Negative		
1	Stable 1 Northern Bačka County	20	7	35.0%	13	11	1/5
2	Stable 2 Northern Bačka County	21	12	57.14%	9	2	0/2
3	Stable 3 South Bačka County	25	16	64.0%	11	12	5/10
4	Novi Bečej – individually raised horses Central Banat County	11	4	36.36%	7	2	0/2
5	Stable 4 South Bačka County	20	10	50.0%	10	3	2/2
6	Stable 5 South Banat County	17	9	52.94%	8	/	/
7	Stable 6 South Banat County	16	6	37.5%	10	/	/
	TOTAL	130	64	49.23%	66	30	3/5 stables 8/21 horses sero-converted

DISCUSSION

This study presents the results of WNV activity in horses in Serbia gained from three different investigations conducted in the period 2007–2013. The seroprevalence varied from 12 % in 2009/2010 to 28.6 % and 49.23 % in 2011 and 2013 respectively [16-18]. The possible reasons for the differences among the tested horses could be found in sample distribution within different herds and horse populations. In our first study, where the WNV seropositivity was estimated at 12 %, equine blood samples were selected randomly. In two other studies, blood samples originated from stables with a higher number of horses. On the other hand, the steady increase in the num-

ber of seropositive horses during this couple of years offers strong evidence that WNV is circulating in the region. The seroconversion detected in 8 horses (*Table 2*) that were negative after the study conducted in 2009/2010 supported our hypothesis of WNV spreading. It is important to emphasize that horse stable 5 (Table 2) is in the same area where the first human WNV clinical case in Serbian WNV 2012 human outbreak were detected. WNV seropositive horses were reported in Croatia, as well as in other European and Mediterranean countries [4, 11, 24, 25].

In 2012, the first outbreak of WNV in humans in Serbia with clinical symptoms was registered. A total of 69 cases were laboratory confirmed, and there were 9 cases with fatal outcome [19-21]. Following the human outbreak in 2012, mosquitoes were collected from 31 municipalities, and WNV RNA was detected in 9.55 % of 314 examined mosquito pools [22]. In the same year, 134 resident wild birds in Serbia were tested for the presence of WNV antibodies, and 7 (7.6 %) blood sera were found to be positive [23]. The West Nile virus was isolated from one Northern goshawk and full genome was sequenced. The phylogenic analyses were classified as lineage 2 WNV strain, closely related to the recent outbreaks in Greece, Italy and Hungary [8, 24, 25].

Although so far no cases of clinical disease in horses have been reported, the epidemiological situation in the neighbouring countries over the past few years warns us that it is necessary to conduct an extensive investigation of WNV infection in Serbia and to implement a special programme of surveillance and disease control.

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Sažetak

Epizootiološko stanje infekcije virusom Zapadnog Nila u konja u Srbiji

Virus Zapadnog Nila (VZN, WNV) prvi je put bio izdvojen u Ugandi 1937. godine. Danas se smatra najrasprostranjenijim flavivirusom u svijetu. Ciklus prijenosa virusa uključuje komarce i ptice, a ljudi i konji krajnji su domaćini. Infekcija kod ljudi i konja često je asimptomatska, ali se mogu javiti klinički znakovi bolesti kao što su groznica i/ili meningitis i encefalitis.

S obzirom na opasnost od širenja infekcije izazvane VZN-om, što je postalo prijetnja za zdravlje životinja i ljudi, proveli smo prvi put u Srbiji serološka istraživanja prisutnosti protutijela za VZN kod zdravih konja. Ukupno je bilo pretraženo 349 uzoraka seruma konja prikupljenih tijekom 2010. iz 28 mjesta u Vojvodini, Beogradu i Šapcu. Uzorci su bili pretraženi validiranim ELISA testom i plak redukcijskim neutralizacijskim testom (PRNT). Rezultati su pokazali da su u 12% (42/349) pretraženih uzoraka ustanovljena protutijela IgG specifična za VZN.

Sljedeće ispitivanje provedeno je 2011. godine, kada je pretraženo 255 sačuvanih uzoraka seruma konja. Ustanovljeno je da je 28,6% (72/255) konja bilo serološki pozitivno na VZN. Za pretraživanje je rabljen komercijalni ELISA test. Odabrani serološki pozitivni uzorci dodatno su bili pretraženi potvrdnim PRNT testom.

Radi procjene prisutnosti i cirkulacije VZN-a kod konja nakon pojave bolesti kod ljudi u Srbiji 2012. godine, sakupljeno je 130 uzoraka seruma konja sa šest ergela i iz jednog naseljenog mjesta u Vojvodini. Uzorci su bili pretraženi komercijalnim ELISA testom, a pozitivni nalazi dodatno su bili potvrđeni i VZN testom. Protutijela specifična za VZN bila su ustanovljena u 49,23% (64/130) konja.

Rezultati naših istraživanja pokazuju da VZN kruži u populaciji konja u Srbiji. Tijekom ljeta 2012. zabilježeni su prvi slučajevi infekcije VZN-om kod ljudi u Srbiji sa smrtnim ishodom, što se nastavilo novim slučajevima i smrtnim ishodima u 2013. godini. Iako do sada nije bilo prijavljenih slučajeva bolesti s kliničkim simptomima kod konja, epidemiološko stanje u susjednim zemljama u posljednjih nekoliko godina upozorava nas da je nužno provesti opsežna istraživanja infekcije VZN-om u Srbiji i uvesti poseban program nadzora i kontrole bolesti.

Ključne riječi: virus Zapadnog Nila; konji; serologija; Srbija.

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MOSQUITOES – VECTORS OF WEST NILE VIRUS IN CROATIA

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Summary

Croatian fauna consists of 50 mosquito species, and 15 of them have medical importance. Only 10 species are capable of transmitting the West Nile Virus (WNV). In Croatia the most capable vector is *Culex pipiens* c. According to species distribution and abundance all of Croatia is a risk area for WNV transmission. *Culex pipiens* c. is the dominant species (share of 5-10%) in eastern Croatia, but *Culex pipiens* c. together with *Aedes albopictus* are now eudominant species in the Adriatic area. Other species have lower vector capacity or they as a species are rare in Croatia. Ecological conditions for mosquito development during 2012 were not favourable and there were not too many mosquitoes in Slavonia, except the area along the Drava River where one flood pulse and one generation of flood mosquitoes was noted. *Culex pipiens* c. breeding sites are various small human made bodies of water and small natural bodies of water. The year 2012 was dry and few specimens of this species were noted. During WNV outbreak in 2012 in Croatia the most probable vector was *Culex pipiens* c.

***Keywords**:* mosquitoes; vectors; West Nile virus; Croatia

INTRODUCTION

The Croatian mosquito fauna consists of 50 species [1,2,3]. Out of these species, 15 have medical significance and only ten of the species are capable of transmitting the West Nile virus. The role of mosquitoes in spreading each arbovirus (viruses transmitted by arthropods), same as with the West Nile virus, is very significant. Mosquitoes are the most significant vectors, but not the only carriers of the West Nile virus. Aside from the mosquitoes this virus vectors can be Phlebotominae, Ceratopogonidae, and Ixodidae. In order for a mosquito to be a vector, some ecological, but also some ethological factors must be present. The virus enters the mosquito during feeding as part of a blood meal from an infected animal (most commonly a

bird). The virus is replicated in the stomach, ovary, nerves, fat tissue, it crosses over to the haemolymph and after that goes to the salivary glands. With new feeding the content of the salivary glands is injected into a host, where the mosquito transfers the virus as an ectoparasite [4]. The main reservoir for the West Nile virus are birds (Centers for Disease Control and Prevention - CDC, 2009.), so, related to that fact, the main vectors are mosquitoes that feed in the blood of birds - the so called ornithophilous species. Considering that mosquitoes are oligophagous (they feed on only a single group of organisms), and they feed multiple times, there is a real possibility that the next meal will be from another species from the group and that the virus will be carried over to that species. There are some extremely ornithophilous species among mosquitoes, that is, monophagous and in that case the transfer of the virus can be from one species of birds to another species of birds. Not all birds are equally exposed and susceptible to viruses. Birds in which the West Nile virus can often be found are from the order Passeriformes (perching birds) and the most common species are house sparrows (*Passer domesticus*) and various species of crows (e.g. *Corvus corone*) and the order Columbiformes (pigeons). Actually the main mechanisms for spreading the West Nile virus are bird migrations. In that context there are two cycles. The rural – sylvatic cycle which consists of the transfer of the virus among wild animals, most commonly swamp birds and ornithophilous mosquitoes. This cycle can be monitored very poorly or not at all. The urban cycle appears between synanthropic, or urban birds and urban mosquitoes. This cycle can be monitored through accidentally infected people or horses. Appearance of diseases caused by the West Nile virus has particularly been registered worldwide in the last twenty years (European Centre of Disease Prevention and Control - ECDC, 2012.).

There are two main parameters which enable the transfer of the disease: fauna and the biology of mosquitoes (vectors) in a specific area, and the biology of pathogens (protozoa and viruses) in that same area. The transfer or spreading of the disease may occur only when optimal conditions for both of these parameters exist. The vector capacity of mosquitoes is different. Some species of mosquitoes are better at transferring viruses while some transfer viruses poorly or not at all. There are about thirty species on the list of species in which the West Nile virus has been found [5]. Of these species, 10 exist in Croatia and are potential vectors, if they come into contact with infected animals.

MOSQUITOES WEST NILE VIRUS VECTORS IN CROATIA

Species of mosquitoes noted in Croatia which can be West Nile virus vectors are: *Culex pipiens* c., *Aedes vexans*, *Aedes cinereus*, *Culex modestus*, *Culex theileri*, *Ochlerota-*

tus caspius, Anopheles plumbeus, Coquillettidia richiardii, Ochlerotatus geniculatus, and *Ochlerotatus cantans* [6,7].

Culex pipiens c.

When describing this species it must be emphasized that it is a polytypic species, which means that the populations of this species are different according to ecological conditions and that they can be differentiated systematically on levels lower than species. (Note on systematics). Considering that, the urban type of this species (*C. pipiens pipiens* biotype *molestus)* exists in locations closer to humans, it represents more of a threat. It has adapted exceptionally well to human settlements, it has breeding sites in canals, barrels, casks, buckets, drains, severage system, etc. As an adult it spends the winter in basements of buildings and houses and in different shalters. According to research conducted over a period of ten years the share of this species in the mosquito fauna in Osijek is 5.86% [10] and in other parts of Slavonia it is 5-10%. Even though it is an ornithophilous species, because it lives so close to humans it is a common vector. The domestic mosquito *Culex pipiens pipiens* biotype *molestus* is the main vector of the West Nile virus, but it also carries the Ockelbo virus, Usutu virus, Sindbis virus, and the Japanese encephalitis virus [4]. Individual units of this species of mosquito are most probably responsible for the transfer of the virus during the appearance of neuroinvasive diseases caused by the West Nile virus in Slavonia during 2012.

Note on sistematics

The complex consists of several species, subspecies, forms, races, physiological variants, or biotypes according to various authors. At present it includes the names *C. pipiens pipiens* Linnaeus, *C. p. pipiens* biotype *molestus* Forskal, *C. p. quinquefasciatus* Say, *C. p. pallens* Coquillett, *C. restuans* Theobald, and *C. torrentium* Martini in the Holarctic as well as two Australian members, *C. australicus* Dobrotworsky and Drummond and *C. globocoxitus* Dobrotworsky. The females of the complex are very difficult to separate in field material. In several reared populations it took eight variables and a discriminant analysis to discern between *pipiens*, *molestus*, and *quinquefasciatus* females and overlapping was considerable. Thus, there is no reliable characteristic yet for discrimination between *pipiens* and *molestus* [4]. For that reasons in this text *Culex pipiens* c. was used to avoid errors.

Aedes vexans

Populations of this species may cause great difficulties for people after spring and summer floods because at those times they reproduce in such large numbers

that, according to estimates, there can be over 100 million larvae per hectare. As its name suggests, it reproduces in flood areas along rivers, swamps, lakes, where there are fluctuations in water levels. It is a good flier and it can fly up to 30 km in search of a blood meal. It is a zoophilic species and it feeds on all animals. If a human crosses its path it will feed on them as well. In nature the flood mosquito can be infected with various arboviruses, some of those are: WEE (West Equine Encephalitis) virus, EEE virus, California encephalitis virus, and in Europe Tahyna virus [4]. In relation to humans it has a significantly smaller vector capacity, since specimens of this species usually able the circulation of the virus among animals. It is a very numerous species in some parts of Croatia.

Aedes cinereus

Specimens of this species can be found in flood areas and the described as a species that appears together with *Aedes vexans*. Its numbers are significantly lower, therefore its vector capacity is lower. It is a moderately abundant species in Croatia.

Culex modestus

The larvae show a preference for shallow sunlit habitats and are frequently found on meadows, in irrigation channels, inundation areas of rivers, or rice fields with rich vegetation. Sometimes can be very numerous in Kopački rit area. The species has repeatedly been reported as an arbovirus vector of two different *Bunyavirus*, Tahyna and Lednice and is also regarded as a potential vector of WNV. Moderate abundant species in Croatia.

Culex theileri

A polycyclic species recorded from a broad range of elevations. The females are zoophilic, but sometimes feed on humans and bite mainly in the open, occasionally in large numbers, also entering houses and other buildings. In South Africa, Sindbis virus and WNV were isolated from wild populations. This species is very rare in Croatia.

Ochlerotatus caspius

This is a polycyclic, halophylic species. Sometimes only one generation per year is produced due to the nature of the breeding site. This species can be very numerous in flooded areas especially in early spring. West Nile virus, Tahyna virus, and the bacterium *Francisella tularensis*, the causative agent of tularemia, could be detected in natural populations of this species. Moderate abundant species in Croatia.

Anopheles plumbeus

Larvae of *A. plumbeus* develop almost exclusively in tree-holes and represents generally rare species. Last decade reports from Germany show that some populations accomodate to new habitat - sewerage system. Although laboratory studies have shown that *A. plumbeus* can successfully be infected with *P. vivax* and *P. falciparum* and that the species is an efficient carrier of malaria, it is considered to be of minor epidemiological importance at the present time because of its ecology. It is also reported as laboratory vector of West Nile Virus. Rare species in Croatia.

Coquillettidia richiardii

Larvae and pupae live submerged and obtain oxygen from the aerenchyma of aquatic plants and move very little. Females infected with WNV, and Omsk haemorrhagic fever virus were detected in wild populations. Moderate abundant species in Croatia.

Ochlerotatus geniculatus

The larvae live in tree-holes at various heights and in open tree stumps of different deciduous trees as *Quercus* sp., *Fagus* sp., *Alnus* sp., *Betula* sp., and *Juglans* sp. They also occur in mixed forests in old trees and can occasionally be found in ground pools. Is is also reported as laboratory vector of West Nile Virus. Rare species in Croatia.

Ochlerotatus cantans

The larvae develop rather early in spring in Southern and Central Europe, Biting females are encountered most abundantly in lowland regions from late March to June. In Croatia this species offten can be find in forests. This species is reported field vector of Tahyna virus, West Nile virus and myxomatosis. Moderate abundant species in Croatia.

MOSQUITOES DURING THE WEST NILE VIRUS OUTBREAK IN SLAVONIA

During 2012 ecological conditions for the development of mosquitoes were not favourable, so the number of mosquitoes in Slavonia was not large, except in the Drava River area where a slightly elevated water level in July resulted in a single generation of flood mosquitoes.

During 2012, which was a drought year, there were not many breeding sites (human made small water bodies - barrels, buckets, drains, septic tanks; natural breeding sites of medium size - canals, depressions), so the number of mosquitoes was small.

During the WNV outbreak (August - September 2012) dry ice baited CDC traps were used to sample mosquitoes in 64 locations in Osijek-Baranja, Vukovar-Srijem, and Brod-Posavina Counties. During the research 5 species of mosquitoes were caught (*Table 1*). Out of the total number of 1785 mosquitoes, 114 were determined as *Culex pipiens* c. which were adequately stored and sent to virus presence analysis. Molecular analysis did not prove the presence of the West Nile virus, but the results point to the possible presence of some other viruses.

Table 1. Number of mosquitoes sampled in eastern Croatia during the West Nile virus outbreak in 2012.

Mosquito species	No.
Aedes vexans	1634
Culex pipiens	114
Ochlerotatus caspius	21
Anopheles maculipennis c.	12
Anopheles hyrcanus	4
Total	1785

INSTEAD OF CONCLUSION

At the moment, the West Nile virus is present in many countries in this part of Europe and its activity was detected in Croatia [8]. The most probable vector which transmits the West Nile virus to the population in Croatia during 2012 is *Culex pipiens* c. The species of *Culex pipiens* c. is the best West Nile virus vector. The species of *Culex pipiens* c. is widely spread in Croatia and in eastern Croatia it is present with the share of 5-10% [9,10] of the mosquito population. Other species represent species with lower vector capacities. The other species of mosquitoes which could be vectors according to the literature have a much lower probability, because some of the species (*Culex theileri, Anopheles plumbeus* and *Ochlerotatus geniculatus*) are rare in Croatia and some are moderately abundant (*Ochlerotatus caspius, Aedes cinereus, Coquillettidia richiardii, Culex modestus* and *Ochlerotatus cantans*). Except for the lower numbers and smaller areas where they are present, it is noted for most of these species that they rarely feed on birds, which considerably reduces the possibility of infection by the West Nile virus.

Although the legal regulations are set up properly, mosquito control in eastern Croatia is not well organised outside large cities (Osijek, Slavonski Brod). Mosquito control should be organised and financed by the local government and smaller units of local government usually cannot afford it. Therefore there is a high probability the virus circulation cycle, birds – mosquitoes – birds, will spread to the human population in the following years.

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Sažetak

Komarci prijenosnici virusa Zapadnog Nila u Hrvatskoj

U Hrvatskoj je zabilježeno 50 vrsta komaraca, a 15 ih ima medicinsko značenje, tj. potencijalni su prijenosnici različitih uzročnika bolesti. Ukupno je deset vrsta potencijalnih prijenosnika virusa Zapadnog Nila. U Hrvatskoj su najbolji vektori jedinke vrste *Culex pipiens* c. Uzmemo li u obzir brojnost i rasprostranjenost te vrste, područje cijele Hrvatske rizično je područje za prijenos virusa Zapadnog Nila. Jedinke te vrste dominantne su (udio 5 – 10% u fauni komaraca) u istočnoj Hrvatskoj, a *Culex pipiens* c., zajedno s jedinkama vrste *Aedes albopictus*, eudominantne su (udio u fauni više od 10%) vrste u jadranskom priobalju. Ostale vrste potencijalni vektori u Hrvatskoj manje su bitne zbog manje brojnosti, ograničenog rasprostranjenja i etoloških odlika. Ekološki uvjeti za razvoj komaraca tijekom 2012. nisu bili pogodni za razvoj većih populacija pojedinih vrsta u Slavoniji. Jedino uz rijeku Dravu zabilježen je veći broj komaraca, što je rezultat nešto povišenog vodostaja rijeke Drave. Legla za jedinke vrste *Culex pipiens* c. različita su manja vodena tijela koja je proizveo čovjek. Godina 2012. bila je suha, tako da je broj komaraca bio relativno malen. Za vrijeme pojave bolesti izazvane virusom Zapadnog Nila zabilježeno je pet vrsta komaraca, a najvjerojatniji vektor bio je *Culex pipiens* c. Ukupno je uhvaćeno 1.785 komaraca, a molekularna analiza na prisutnost virusa Zapadnog Nila obavljena je na 114 jedinki vrste *Culex pipiens* c. Virus nije potvrđen kod komaraca.

Ključne riječi: komarci; vektori; virus Zapadnog Nila; Hrvatska.

WEST NILE VIRUS INFECTIONS IN EUROPE – GENERAL FEATURES

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Summary

In the past West Nile virus (WNV) infections occurred only sporadically in Europe, limited in time and geographic distribution. This changed dramatically since the emergence of an exotic lineage 2 WNV strain in Hungary in 2004 [Bakonyi et al., Emerg. Infect. Dis. 12, 618-623 (2006)]. Following a few years of adaptation, this virus strain dispersed widely in 2008, and was identified all over Hungary and in the eastern part of Austria [Wodak et al., Vet. Microbiol. 149, 358-366 (2011)]. Most likely during that time this WNV lineage also spread to Croatia, Slovenia and Serbia. In 2010, a clinically severe outbreak occurred in the Thessaloniki area of Greece with more than 200 human neuroinvasive cases and more than 30 deaths. Genetic analysis demonstrated the above-mentioned lineage 2 WNV as the causative agent, however with an amino acid exchange, which might have been responsible for the increased neuroinvasiveness [Papa et al., Emerg. Infect. Dis. 17, 920-922 (2011)]. In 2012, overt West Nile disease in humans and animals was also reported from several Balkan states.

Since 2008 widespread outbreaks of lineage 1 WNV have been reported in northern Italy as well as outbreaks of a different type of a lineage 2 WNV (Volgograd strain) in Romania.

We demonstrated that the virus is overwintering in mosquitoes in outbreak areas, indicating that central, southern and eastern Europe must be aware of further WNV outbreaks in the future.

General features of WNV infections and an overview of WNV epidemiology in Europe are presented.

Keywords: West Nile virus; Europe; epidemiology.

Sažetak

Infekcija virusom Zapadnog Nila u Europi – osnovna obilježja

U prošlosti se infekcija virusom Zapadnog Nila (VZN) u Europi javljala sporadično te vremenski i geografski ograničeno. To se stubokom promijenilo otkad se 2004. godine u Mađarskoj pojavila egzotična linija 2 VZN-a [Bakonyi et al., Emerg. Infect. Dis. 12, 618-623 (2006.)]. Nakon pet godina prilagodbe, soj te linije nadaleko se proširio te je 2008. dokazan na cijelom području Mađarske i u istočnom dijelu Austrije [Wodak et al., Vet. Microbiol. 149, 358-366 (2011.)]. Ta se linija vjerojatno u tom razdoblju proširila i na Hrvatsku, Sloveniju i Srbiju. Klinički teški oblik infekcije VZN-om pojavio se 2010. u Grčkoj s više od 200 neuroinvazivnih slučajeva i više od 30 umrlih. Genetska analiza pokazala je da je uzročnik te epidemije bila spomenuta linija 2 VZN-a s izmijenjenim aminokiselinskim sastavom, što je vjerojatno bio uzrok njezine pojačane neuroinvazivnosti [Papa et al., Emerg. Infect. Dis. 17, 920-922 (2011.)]. Kliničko očitovanje infekcije VZN-om zabilježeno je 2012. u ljudi i životinja u nekoliko balkanskih država. Od 2008. zabilježeno je nekoliko pojava infekcije linijom 1 VZN-om u sjevernoj Italiji te pojava različitih tipova linije 2 VZN-a (soj Volgograd) u Rumunjskoj. Dokazali smo da virus prezimljuje u komaraca na područjima gdje se trajno javlja, što upućuje na zaključak da se u budućnosti mogu očekivati nove pojave infekcije VZN-om u središnjim, južnim i istočnim dijelovima Europe.

U izlaganju su iznesena opća obilježja infekcije VZN-om i općenit pregled epidemiologije te infekcije u Europi.

Ključne riječi: virus Zapadnog Nila; Europa; epidemiologija.

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WEST NILE VIRUS IN ITALY, FIVE YEARS OF EPIDEMIC

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Summary

West Nile virus (WNV) first appeared in Italy in 1998. Although the infection caused severe disease and deaths in horses, no cases were reported in humans at that time. Since 2008, the Italian epidemiological scenario of WNV has been dramatically changing with neurological diseases being increasingly observed in both, humans and horses. In order to monitor and control the WNV circulation, the Italian government implemented both a national serological, entomological and virological surveillance program and a national plan for West Nile Neuroinvasive Disease. Within the framework of WNV surveillance activities, it was possible to detect and monitor the progression and evolution of different strains of WNV from their entry in the North Eastern part to their spread into the South and main Islands of Italy. In some areas co-circulation of WNV strains belonging to lineage 1 and 2 was also evidenced. The presentation will describe the main features observed during these last five years of outbreak.

Keywords: West Nile virus; Italy; evolution; epidemiology.

Sažetak

Pet godina epidemije uzrokovane virusom Zapadnog Nila u Italiji

Virus Zapadnog Nila (VZN) prvi je put u Italiji bio dokazan 1998. godine. Premda je tada uzrokovao tešku bolest kod konja i njihovo ugibanje, bolest nije bila zabilježena kod ljudi. Epidemiologija infekcije virusom Zapadnog Nila u Italiji znatno se promijenila nakon 2008. godine. Otada se sve češće javlja s nervnim poremećajima u ljudi i konja. Radi promatranja kruženja i kontrole VZN-a, u Italiji je na državnoj razini donesen nacionalni plan za provođenje programa seroloških, entomoloških i viroloških istraživanja neuroinvazivne bolesti uzrokovane virusom Zapadnog Nila. U sklopu promatranja proširenosti infekcije VZN-om proučavana je evolucija različitih sojeva virusa od njihove pojave u sjevernim i istočnim područjima Italije do širenja na jug i glavne talijanske otoke. U nekim područjima

dokazana je istodobna aktivnost sojeva linije 1 i linije 2 VZN-a. U izlaganju su iznesena osnovna epidemiološka obilježja infekcije posljednjih pet godina.

Ključne riječi: virus Zapadnog Nila; Italija; evolucija; epidemiologija.

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CHARACTERIZATION OF WEST NILE VIRUS OUTBREAKS IN HUNGARY

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Summary

The occurrence the West Nile virus (WNV) in Hungary was first reported on the basis of serological studies on geese in 1969. However, clinical manifestation of the virus infection has never been observed in the country until 2003. In that year an outbreak of encephalitis occurred in a goose flock at the southern region of Hungary. A lineage 1 WNV strain was detected in the brain of the affected birds. One year later an exotic, lineage 2 strain of WNV emerged in south-eastern Hungary. This virus strain differed from the strains distributed in Europe, Asia, America and Australia; its closest relatives were isolated in the sub-Saharan region of Africa. It is likely that migratory birds introduced the strain to Hungary from their African wintering places. The first neurological and lethal cases were observed in goshawks (*Accipiter gentilis*) in 2004, and the virus was subsequently detected in 2005 in diseased or dead goshawks, sparrow-hawks (*Accipiter nisus*) and in a sheep. The cases were observed in a close geographic proximity (approx. 30 km distance from each other). In 2007 we detected WNV infections in three goshawk and three red-footed falcon (*Falco vespertinus*) samples collected in the territories of the Körös-Maros National Park and the Hortobágy National Park. A moderate geographic spread of the virus was observed (approximately 80 km distance from the location of the year 2004-2005 cases). An unexpected and explosive geographic spread of the virus strain was detected in 2008. We found 25 WNV positive wild bird cases. Goshawks were the most seriously involved (17 diagnosed cases) but other species, mainly birds of prey were also found WNV infected. The virus strain emerged in the central and western regions of Hungary and in the eastern part of Austria. In 2009 we detected WNV in 16 wild bird specimens. The highest number of cases was again diagnosed in goshawks, but small songbirds were also found positive. Cases were distributed over the whole territory of Hungary and in the eastern regions of Austria. In 2010, a lower intensity of WNV activity was observed in Hungary, compared to the previous two years. Three wild bird samples were found positive for WNV. In 2011 further four WNV infections were diagnosed in wild birds. Genetic comparisons of the viruses detected between 2004 and 2011 revealed a high level of genetic relatedness of the strains, which indicate that the previously exotic WNV

strain, which was introduced to Hungary around 2004, established itself in the country and became a resident pathogen.

Besides wild birds, WNV-associated central nervous illnesses were diagnosed in horses (2007: 1 case, 2008: 12 cases, 2009: 5 cases, 2010: 5 cases, 2011: 4 cases, and 2012: 3 cases. Many of these horses died due to encephalomyelitis), and in humans (2008: 19 cases, 2009: 7 cases, 2010: 19 cases, 2011: 6 cases. 2012: 7 cases. One case in 2010 was fatal). We have also detected the virus in mosquito vectors. Serological investigations were made on serum samples of wild birds and horses for the detection of anti-WNV antibodies, in order to estimate the frequencies of subclinical infections. Antibodies were detected in 10-40% of the serum samples, which indicate a relatively high infection rate. The frequent and widespread subclinical infection is a possible explanation for the reduced number of clinical cases in Hungary in 2010, 2011, and 2012, compared to 2008 and 2009. Nevertheless, the WNV strain emerged in 2010 in the north-eastern part of Greece and caused a serious outbreak in people with several encephalitic cases and a high fatality rate. The same virus strain was also detected in a human case in Italy in 2011, was well as in Serbia in 2011 (in mosquitoes) and in 2012 (in horses and in humans). Our genetic investigations demonstrated that the strain is practically identical with the one that emerged in Hungary and which has been circulating in the country since.

Keywords: West Nile virus; Hungary; horses; wild birds.

Sažetak

Osobitosti infekcije virusom Zapadnog Nila u Mađarskoj

Pojava virusa zapadnog Nila (VZN) u Mađarskoj prvi je put bila zabilježena na osnovi seroloških istraživanja u gusaka 1969. godine. Međutim kliničko očitovanje zaraze nije bilo ustanovljeno sve do 2003. Te se godine pojavio encefalitis u jatu gusaka u južnom dijelu Mađarske. U tkivu mozga zaraženih gusaka ustanovljena je linija 1 virusa Zapadnog Nila. Sljedeće godine pojavila se egzotična linija 2 VZN-a u jugoistočnom dijelu Mađarske. Taj soj bio je različit od sojeva virusa koji su kružili u Europi, Aziji, Americi i Australiji. Njegovi najbliži srodnici bili su izdvojeni u supsaharskom području Afrike. Soj su u Mađarsku vjerojatno prenijele ptice selice iz afričkih područja gdje one prezimljuju. Prvi neurološki slučajevi i uginuća zapaženi su u jastreba *(Accipiter gentilis)* 2004., a virus je bio dokazan i 2005. u oboljelih ili uginulih jastrebova, kobaca *(Accipiter nisus)* te u ovaca. Svi slučajevi bili su ustanovljeni na uskom geografskom području (u razmaku oko 30 km jedan od drugoga). Godine 2007. infekcija VZN-om bila je dokazana u tri jastreba i tri sokola *(Falco vespertinus)* na području Nacionalnog parka Körös-Maros i Nacionalnog parka Hortobágy. Tada je primijećena velika geografska proširenost virusa (na udaljenosti oko 80 km od mjesta gdje su bili utvrđeni slučajevi 2004. i 2005.). Neočekivano i eksplozivno širenje virusa ustanovljeno je 2008. Virus je tada bio dokazan u 25 divljih ptica. Najčešće se bolest javljala u jastrebova (17 dijagnosticiranih slučajeva), ali su bile zahvaćene i druge vrste, pretežito grabljivice.

Virus se pojavio u središnjim i zapadnim područjima Mađarske te istočnom dijelu Austrije. Godine 2009. VZN je bio dokazan u 16 uzoraka divljih ptica. Najviše slučajeva ponovo je bilo dokazano u jastrebova, ali i u malih ptica pjevica. Zaraza se proširila na cijelo područje Mađarske i na istočni dio Austrije. Godine 2010. ustanovljena je smanjena aktivnost VZN-a u odnosu na prijašnje godine. Svega tri uzorka divljih ptica bila su pozitivna na virus. Godine 2011. virus je bio dokazan u daljnja četiri slučaja. Usporedba genetskih svojstava sojeva dokazanih od 2004. do 2011. pokazala je njihovu veliku genetsku srodnost, što upućuje na zaključak da se prethodno egzotični virusni soj, koji je bio unesen u Mađarsku oko 2004., tu udomaćio i postao prirođen mađarskom području.

Osim u divljih ptica VZN je bio povezan i s dijagnosticiranim poremećajima središnjeg živčanog sustava u konja (2007.: jedan oboljeli, 2008.: dvanaest oboljelih, 2009.: pet oboljelih, 2010.: pet oboljelih, 2011.: četiri oboljela i 2012.: tri oboljela). Mnogi od tih konja uginuli su pod znakovima encefalomijelitisa. Infekcija je bila dijagnosticirana i u ljudi (2008.: 19 oboljelih, 2009.: 7 oboljelih, 2010.: 19 oboljelih, 2011.: 6 oboljelih, 2012.: 7 oboljelih. Godine 2010. jedan je pacijent umro.).

U Mađarskoj je virus bio dokazan i u komaraca, njegovih prenositelja. Poduzeta su bila i serološka istraživanja u divljih ptica i konja na prisutnost specifičnih protutijela radi procjene učestalosti supkliničkih infekcija. Protutijela su bila dokazana u 10 – 40% pretraženih uzoraka seruma, što govori o relativno visokoj stopi infekcije. Sve većom učestalošću i proširenošću supkliničke infekcije mogao bi se objasniti smanjeni broj kliničkih slučajeva u Mađarskoj 2010., 2011. i 2012. godine u odnosu na 2008. i 2009. Soj VZN-a koji se 2010. pojavio u sjeveroistočnom dijelu Grčke uzrokovao je teški oblik encefalitisa s visokom smrtnošću u ljudi. Isti virus bio je dokazan i u ljudi u Italiji 2011., zatim u Srbiji 2011. (u komaraca) i 2012. u konja i ljudi. Naša genetska istraživanja pokazala su da je taj soj zapravo identičan soju koji je bio dokazan u Mađarskoj, a sada kruži cijelom zemljom.

Ključne riječi: virus Zapadnog Nila; Mađarska; konji; divlje ptice.

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Symposium

CORNEAL TRANSPLANTATION AND EYE BANKING

Guest Editor

Iva Dekaris

CORNEAL TRANSPLANTATION AND EYE BANKING

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Scientific Symposium "Corneal Transplantation and Eye Banking" has been organized by The Department of Medical Sciences of the Croatian Academy of Sciences and Arts and Special Eye Hospital "Svjetlost" of the School of Medicine in Rijeka, University of Rijeka, in the Palace of the Academy in January 2013. Just after this symposium, and under the Auspices of the Croatian Academy of Sciences and Arts, Special Eye Hospital "Svjetlost" and Croatian Society for Cataract and Refractive Surgery organized the XXV Annual Meeting of the European Eye Bank Association (EEBA).

The symposium in the Academy and the Annual EEBA Meeting were dedicated to the latest developments in the field of corneal transplantation and eye banking. The invited lectures where presented by the most prominent experts around the globe: President and Past-President of the Eye Bank Association of America (K. Corcoran and prof. M. Macsai), the President of the Association of Eye Banks of Asia (prof. D. Tan), the President of the European Eye Bank Association (prof. I. Dekaris), the President of the Italian Society for Corneal Transplantation (prof. M. Busin), Chairman of the Eye Hospital of the University in Freiburg (prof. T. Reinhard), the member of the Bulgarian Academy of Sciences (prof. P. Vassileva) and Chair of the Cornea Section of the European Association for Vision and Research (dr. T. Fuchsluger). The topics that were covered were: new trends in corneal transplantation in USA, innovations in lamellar corneal transplantation, strategies of limbal stem cell transplantation, gene and cell therapy to protect corneal cells, new surgical approach in high-risk corneal grafts and novel treatments for herpetic eye disease.

The importance of the Symposium and Annual Meeting lays in the fact that approximately 20 million people are blind due to some kind of corneal disease, and most of those patients can be cured by corneal transplantation. The major step forward in the quality and quantity of corneal transplantations came with a foun-

dation of eye banks. Eye banks are in fact specialized laboratories in which donor corneal tissue can be tested for its safety and examined under the microscope for its quality. In all countries where eye bankers and corneal surgeons had good collaboration, waiting lists for corneal transplantation were "cleaned" and corneal blindness is nowadays successfully treated. However, although corneal transplantation is the most commonly performed transplantation procedure with over 100 000 procedures performed yearly, and in western countries it is a planned surgery; there are many regions in the world with significant lack of donor corneal tissue for transplantation and very long waiting lists. Like every other field of medicine, corneal surgical procedures and eye banking methods had continuously developed, bringing us to a completely new era of corneal transplantation. Penetrating or full-thickness corneal transplantation was a gold surgical standard for over 100 years, but in a last decade novel surgical techniques were adopted by corneal surgeons, and number of so-called lamellar transplantation is rapidly increasing. In lamellar transplantation only the diseased part of the cornea is grafted, instead of a full-thickness cornea. It has the advantage that it is a safer surgery for patient's eye, it brings much quicker visual rehabilitation, and theoretically one donor cornea can be used for two surgeries. These changes in surgical technique had a great impact on the everyday work of eye-bankers as well, since the preparation of lamellar corneal grafts can be made by eye-bank staff and not only by the surgeon in the operating room. Therefore, the "old" role of eye bankers, which was to take care of the quality and safety of a donor tissue, has been expanded to tissue preparation for the surgery. The Annual EEBA Meeting which is organized each year in a different European country, is the main scientific event for the exchange of knowledge and gathering of different specialists involved in eye banking and corneal transplantation. Today, European Eye Bank Association (EEBA) unites more then 80 eye banks from 24 European countries and 10 international eye-banks. In a last 5 years, European eye banks were processing over 30 000 donor corneas yearly (both those for penetrating and lamellar transplantation), providing enough donor corneal tissue for European needs and helping patients with corneal diseases to regain their site. During Meetings in Zagreb, they have exchanged their knowledge through 12 invited lectures and 40 scientific and professional papers organized in 5 scientific sessions. Over 200 attendees from more then 20 countries have participated at Symposium and EEBA Meeting, and many of them could benefit not only from a scientific sessions, but also from the "wet-lab" which was organized to train younger colleagues on a real-eye models on how to perform lamellar corneal transplantation.

Figure 1. President of the Croatian Academy of Sciences and Arts, Zvonko Kusić, is addressing the audience at the Opening of the Scientific Symposium „Corneal Transplantation and Eye Banking" in the Palace of the Academy in January 2013. Speakers at Symposium are sitting in the first row (from right to left): prof. M. Macsai (USA), prof. D. Tan (Singapore), dr. T. Fuchsluger and prof. T. Reinhard (Germany), prof. I. Dekaris (Croatia); together with Secretary of the Department of Medical Sciences, Marko Pećina and Secretary General of the Academy, Pavao Rudan.

Figure 2. Opening ceremony of the XXV EEBA Meeting on January 2013 in Zagreb. From left to right: prof. Nikica Gabrić, Head of Special Eye Hospital "Svjetlost", prof. Rajko Ostojić, Croatian Minister of Health. and Zvonko Kusić – President of the Croatian Academy of Sciences and Arts

Figure 3. Closing ceremony of XXV EEBA Meeting – transfer of the flag to the organizer of the next EEBA Meeting in Lausanne in 2014 (prof. Iva Dekaris - left and assoc. prof. Francois Majo - right).

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