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QUANTIFICATION OF 11-DEHYDRO-THROMBOXANE B2 IN URINE - ADVISABLE LABORATORY METHOD FOR DETECTION ASPIRIN EFFICACY OR RESISTANCE?

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A b s t r a c t

Increased incidence of cardiovascular events in patients with the insufficient inhibition of platelet function tests has been shown in many studies. Therefore, identification of patients with high residual platelet reactivity, despite of receiving aspirin in long-term prevention may be useful to predict their risk of atherothrombotic events. Historical gold standard test the (optical) light transmission aggregometry (LTA) has got specific limitations. The urinary concentrations of the stable TxA2 metabolite, 11-dehydro-thromboxane B2 (11-dhTxB2) represent time-integrated index of TxA2 biosynthesis *in vivo*. The TxA2 pathway is essential for the full aggregation response of platelets and it is target pathway of aspirin. Therefore, the urinary 11-dhTxB2 could be the optimal test for detection of aspirin effectivity or resistance. The aim of our study was the comparison of traditional LTA after stimulation by arachidonic acid (AA) with concentrations of urine 11-dhTxB2 in 33 healthy volunteers. Examination by LTA showed decreased ability to aggregation of platelets under 20% after stimulation by AA in 32 of 33 healthy subjects, who received 100 mg aspirin daily during 3 days. In one man the aggregability of platelets did not decreased enough, till the dose of aspirin was increased to 200 mg daily. The concentration of urine 11-dhTXB2 decreased under the level of cutoff in all 33 examined healthy subjects after 3 days of receiving aspirin. The correlation between platelet aggregation and concentrations of the urine 11-dhTxB2 after receiving aspirin was not statistically significant despite of good "treatment" response reflected by both methods. It would not make it possible to perform rapid and easy screening of platelet function during the prophylaxis and treatment with aspirin yet. Further research is necessary to find if it would be available to screen aspirin resistance and in some cases, to predict the risk of atherothrombotic events in patients with cardiovascular diseases.

Key words: (optical) light transmission aggregometry - urine 11-dehydro-thromboxane B2 - aspirin resistance – atherothrombotic events

INTRODUCTION

Drugs that inhibit platelet function are widely used to decrease the risk of occlusive arterial events in patients with atherosclerosis. Aspirin selectively affects a single pathway of platelet activation: thromboxane A2 (TxA2) pathway by irreversibly inhibiting COX-1, resulting in inhibition of TxA2 production. The TxA2 pathway contributes to the amplification of platelet activation and it is essential for the full aggregation response of platelets (1). The loss of the potent platelet activator drastically reduces platelet aggregation (2). Not all patients have benefit from the protection that this drug provides and they are exposed to a high risk of atherothrombotic events. Previous studies have estimated that about 5.5 – 56.8 % of the population is aspirin resistant (2). In recent years the issue of resistance to aspirin has been largely emphasized in the medical literature (3, 10, 11, 13). Despite several studies published on this issue, its definition, diagnosis, causes and clinical consequences are still uncertain. The mechanism of resistance remains incompletely defined. Patients receiving antiplatelet agents exhibit a wide variability in response: some are good responders, others are intermediate responders and a few are low responders or resistant (non-responders). This interindividual variation may be the result of acquired factors that interfere with aspirin function or genetic determinants (5, 6, 7). The demonstration that some patients

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may be “resistant” or “poor responders” to the pharmacological effect of antiplatelet drugs, has prompted the need of laboratory monitoring of antiplatelet therapy. The ideal test should be inexpensive, easy to perform, quick, reproducible, accurate, well standardized, so that the patient on antiplatelet treatment could be monitored in any laboratory, obtaining comparable results (1). We compared the detection methods for aspirin resistance using traditional platelet aggregometry and concentrations of urine 11-dehydro-thromboxane B2 (11-dhTxB2).

METHODS

33 healthy volunteers were included in the study. They were examined twice, first before receiving any medication, then they received 100 mg aspirin for three days and on the third day the same examination was performed. The aggregation of platelets was examined in these subjects by (optical) light transmission aggregometer (LTA) PACKS-4 (Helena Laboratories) after stimulation by arachidonic acid (AA) to evaluate the effect of aspirin. Whole blood tubes were centrifugated at 179g for 15 min. to prepare platelet-rich plasma (PRP). The plasma was further centrifugated at 2500g for 15 min. to recover platelet-poor plasma (PPP). Platelet counts of PRP were adjusted to at least $200x10^9$/l with autologous PPP. Platelets were stimulated with 0.5 mmol/l AA. Aggregation was assessed as the maximum percent change in light transmittance from baseline, with PPP used as a 100% transmittance reference. Aspirin resistance was defined as aggregation of ≥ 20%. Sufficient therapeutic response was characterized by decreased aggregation of platelets after stimulation by AA under 20%. All assays were completed within 4 h after blood sampling. Simultaneously concentrations of urine 11-dhTxB2 and urine creatinine were measured by separate assays. The AspirinWorks® Test Kit (Corgenix) is a quatitative enzyme-linked immunoassay (ELISA) to determine levels of 11-dhTxB2 in human urine, which aids in the determination of platelet response to aspirin in patients. Diluted samples with purified 11-dhTxB2 conjugated to alkaline phophatase, and purified mouse monoclonal antibody directed to 11-dhTxB2 are combined and incubated in microwells coated with polyclonal anti-mouse antibody. Incubation allows the endogenous 11-dhTxB2 present in samples to compete with purified AP-conjugated 11-dhTxB2 for binding to the mouse monoclonal anti - 11-dhTxB2. The monoclonal antibody then binds to the polyclonal anti-mouse antibody coated on microtiter plate. The complex formed on the plate is composed of monoclonal antibody and endogenous or AP-conjugated 11-dhTxB2. After the removal of unbound complexes by washing, the bound AP-11-dhTxB2 conjugate is assayed by the addition of paranitrophenylphosphate (pNPP) chromogenic substrate. Color develops in the wells at the intensity inversely proportional to the sample urine concentration of 11-dhTxB2, and is read on a spectrophotometer at 405 nm. Results (in pg/ml) are calculated against a reference curve constructed from the reference solution provided in the kit. Final results are reported as pg 11-dhTxB2 per mg creatinine to normalize results for urine concentration. Results are presented as positive or negative, based on cutoff of 1500 pg 11-dhTxB2 per mg urinary creatinine. Statistical analysis and calculation of the statistical significance were done with SPSS 12.1 software using Spearman rank correlation to compare results of platelet function between platelet aggregation and concentrations of urine 11-dhTxB2.

RESULTS

Examination by LTA showed decreased ability to aggregation of platelets under 20% after stimulation by AA in 32 from 33 healthy subjects, who received 100 mg aspirin for 3 days. Overall, platelet aggregation to AA was 82.18 ± 4.02% (median 81) before and 10.39 ± 4.91%

(median 10) after receiving aspirin for 3 days (Fig.1). Ratio represents 81/10 (%). In one man the aggregability of platelets did not decreased enough, till the dose of aspirin was increased to 200 mg per day. Ratio before and after receiving aspirin 200 mg for 3 days was 35/15 (%). In this person higher dosage of aspirin was needed to obtain adequate pharmacological effect of aspirin for inhibition of platelets. The concentration of urine 11-dhTXB2 decreased under the level of cutoff in all 33 examined healthy subjects after 3 days of receiving aspirin. Ratio before and after receiving aspirin for 3 days represents 1980/364 (pg/ml creatinine) (Fig.2). These data reflect good response to "treatment" by aspirin. The correlation between platelet aggregation and concentrations of the urine 11-dhTxB2 after receiving aspirin (100 mg) for 3 days was not statistically significant, correlation coefficient was r = - 0. 024 (p>0.001).

Tab. 1. The comparison data of platelet aggregation and urine 11-dehydro-Thromboxane B2 concentrations before and after receiving aspirin

	Aggregation (%) n: 32	**Concentration of urine 11-dhTxB2** (pg/ml creatinine) n: 33
before aspirin	median 81 (76 – 91)	median 1980 (1218 - 5500)
after aspirin (100 mg daily during 3 days)	median 10 (0 - 25)	median 364 (193 - 1265)

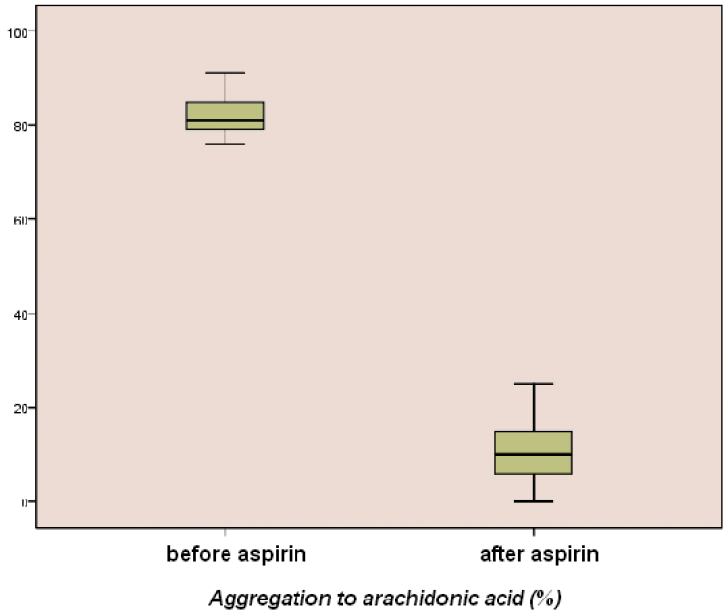

Fig. 1. Platelet aggregation to arachidonic acid before and after receiving aspirin

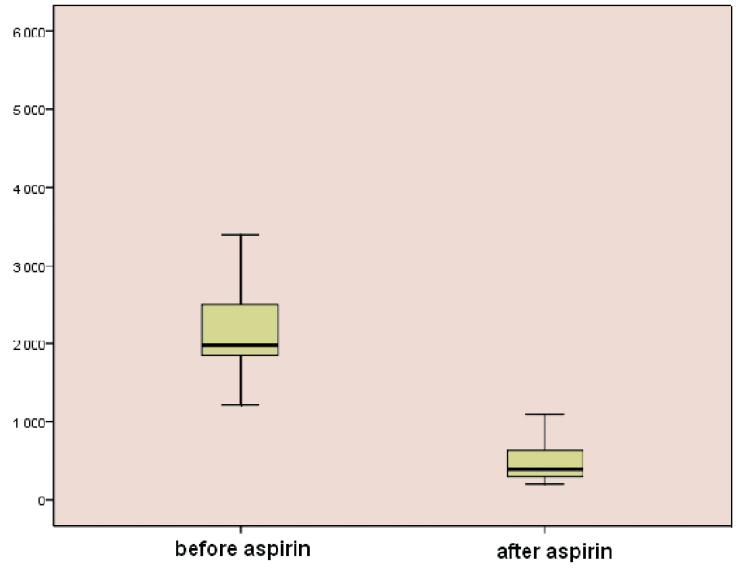

Fig. 2. Concentrations of urine 11-dehydro-thromboxane B2 in healthy persons before and after receiving aspirin

DISCUSSION

Antiplatelet drugs, including aspirin, are beneficial in the prevention of coronary artery disease, ischemic stroke, and peripheral artery disease. However, there is well-documented variability between patients´ responses to those drugs (with regard to laboratory test) (2). The patients who experience atherothrombotic events while on anti-platelet treatment, can be "resistant" to the drug or "treatment failure" (called also clinical resistance) is the reason. The term "resistance" to a drug is used when a drug is unable to hit its pharmacological target, because of inability to reach it or to alterations of target, so resistance to aspirin should be limited to situations in which aspirin is unable to inhibit COX-1 dependent TxA2 production and consequently TxA2-dependent platelet functions (1). Inhibition of this pathway of platelet activation negatively affects not only thrombus formation *in vivo*, but also platelet activation *in vitro*. Global tests measuring platelet aggregate formation *in vitro* on antiplatelet treatment may identify patients with high residual platelet reactivity, but they do not necessarily identify patients who are resistant to antiplatelet drug (1). Only the use of specific tests that measure the pharmacological effect of the antiplatelet drug will clarify whether their platelet hyperreactivity is due to insufficient pharmacological effect of the drug or to other causes. Therefore many studies used various techniques to measure platelet function *in vitro* in order to evaluate degree of its inhibition by antiplatelet treatment. Increased incidence of cardiovascular events in patients with insufficient inhibition of platelet function tests has been shown in many studies (1). Therefore, identification of patients with high residual platelet reactivity may be useful to predict their risk of atherothrombotic events and could improve outcomes. However, many studies still need to be carried out to identify the ideal laboratory test and to answer basic question on its clinical utility and cost-effectiveness, before monitoring antiplatelet therapy can be recommended in clinical practice.

There are various types of tests: LTA, impedance aggregometry, whole blood platelet aggregation, PFA-100®, RPFA – Verify-Now (The Ultegra Rapid Platelet Function Assay), CPA (The Cone-and-Platelet Analyzer), flow cytometry, thrombelastography (TEG), serum thromboxane B2 (TxB2). LTA is the historical gold standard test to measure antiplatelet effects of aspirin (2). It measures the increase in light transmission through a platelet suspension that occurs when platelets aggregate in response to an agonist, but it is neighter adequate nor ideal for measuring the pharmacological effect of aspirin, because this method has got specific limitations. It is time-consuming, it should be performed only in specialized laboratories, there are many pre-analytical and analytical variables affecting the results, accuracy and reproducibility of this technique is very poor, the results can be hardly compared with those obtained in different laboratories because of lack of standardization and finally they depend on the type and concentration of agonist used, and the type of anticoagulant used for blood collection (3). Studies showed that although platelet COX-1 activity seemed to be uniformly inhibited in all studied patients, LTA studies showed great inter-individual differences (14). This *in vitro* method is criticized by some authors because of non-physiologic conditions (22). To obtain PRP, blood is usually taken on an anticoagulant containing sodium citrate, which decreased calcium ions´ concentration about 20-fold and the platelet suspension is devoid of other blood elements, which *in vivo* interact with platelets. In addition, platelet clumbing depend on interplay of various external and secreted agonists, therefore results may be variable. For these reasons LTA may not demonstrate the real ability of platelets to form aggregates and reflect the inhibitory action of aspirin on platelet function (22).

The aim of our study was to verify the usefulness and accuracy of new method for evaluation of aspirin effect on platelet aggregation by comparison of the arachidonic acid – stimulated platelet aggregation as a standard method with concentrations of urine 11-dhTxB2 as a new method in healthy subjects receiving aspirin (100 mg) for 3 days. Platelet COX-1 is generally accepted as the major source of TxA2 in humans, therefore aspirin effectivity or resistance can be diagnosed in the laboratory by measurement of platelet TxA2 production or thromboxane-dependent platelet function (3, 22). Serum thromboxane B2 (TxB2) reflects the total capacity of platelets to synthesize TxA2, of which it is a metabolite, therefore it is the most specific test to measure the pharmacological effect of aspirin (10), but it is not stable enough. The urinary concentrations of the TxA2 metabolite, 11-dhTxB2 represent time-integrated index of TxA2 biosynthesis *in vivo*. Because it is not formed in the kidney, detection of its level reflects systemic formation of TxA2, which largely occurs in platelets. It is stable metabolite of TxA2, therefore it allows good detection. Production of TxA2 is not typical only for platelet. Usually relatively small amounts of TxA2 could be produced by aspirin-insensitive mechanisms, by COX-2 in polymorphonuclear leukocytes and possibly in very young platelets or interaction of platelet with red blood cells and leukocytes, with formation of platelet-leukocyte aggregates may also lead to aspirin-insensitive TxA2 formation (22). In some pathological conditions (inflammatory diseases etc.) about 30% of its urinary metabolite may derive from extra-platelet sources (15). However, this method does not seem to be highly specific for monitoring the effect of aspirin on platelet COX-1, sub-optimal reduction of urinary 11-dh-TxB2 concentrations during aspirin treatment and lack of inhibition by aspirin of TxA2 biosynthesis were associated with reduced cardioprotective effect of aspirin in many studies (14, 16, 17, 18). The biggest was HOPE (Heart Outcomes Prevention Evaluation) trial, 970 patients enrolled and reported statistically significantly higher risk for a composite outcome of myocardial infarction, cardiovascular death or stroke during following 5 years in patients with urinary 11-dhTxB2 concentration in the highest quartile compared with those in the lowest quartile (22). So aspirin resistance may be defined as laboratory resistance and clinical resistance. Aspirin resistance, as determined by specific tests (e.g. serum thromboxane B2), appears to be rare. Many studies, similar to our results, showed that the assessment of aspirin resistance is highly assay-dependent and the overall variability was most notable for assays that did not use arachidonic acid as agonist (4).

Incidence varies between 1-1,7% in studies that measured TxB2 levels (14, 19, 20) and less than 1% in studies that used arachidonic acid – induced platelet aggregation (12, 21).

Our findings in healthy volunteers, that there is not statistically significant correlation between the historic gold standard test represents by LTA and concentrations of urine 11-dhTxB2 confirm these methods depend on different influences. They provide the evidence that effect of aspirin on ability of platelet to aggregate *in vivo* might differ from those observed *in vitro* despite of the good "treatment" response reflected by both methods.

In conclusion, laboratory aspirin resistance remains a clinically important phenomenon and patients identified as subjects with aspirin resistance are at increased risk of recurrent cardiovascular events, but no study was able to show which platelet function test optimally identifies treatment response on aspirin and aspirin resistance patients yet. Our study indicates that the results were variable compared between traditional LTA and concentrations of urine 11-dhTxB2 to detect aspirin response and it would not make it possible to perform rapid and easy screening of platelet function related to receiving aspirin yet. Further research is necessary to find, if it would be available to screen aspirin resistance and in some cases, to predict the risk of atherothrombotic events in patients with cardiovascular diseases.

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DISTURBANCES OF GLUCOSE AND LIPID METABOLISM ASSOCIATED WITH ANTIPSYCHOTIC TREATMENT

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Abstract

Antipsychotic drugs were discovered in the 1950's and represent an important class of psychotropic medications. They are the treatment-of-choice for schizophrenia and similar severe mental disorders but are also widely used outside of the psychiatric specialty for the treatment of a broad range of symptoms.

However, a range of evidence, from case reports to controlled studies, indicates that some of these agents are associated with adverse changes in lipid and glucose metabolism, which, in some cases, can develop independently of weight gain. Besides, several reports suggest, that patients with mental disorders such as schizophrenia have an increased prevalence of metabolic abnormalities prior to initiation of antipsychotic therapy, so it can not be entirely explained as impact of antipsychotic treatment. Also lifestyle factors are likely to play an important role in development of these disturbances.

Individuals with mental illnesses possess a substantial burden of metabolic morbidity and die at an earlier age from these conditions versus general population (1). Therefore, physical health monitoring, especially in long-term antipsychotic treatment, is essential for early detection of the metabolic disturbances, which should be followed by subsequent appropriate interventions.

This article summarizes attainable informations about the potential impact of antipsychotics on the glucose homeostatic network and lipid milieu. It also provides results of our retrospective study and recommendations for the systematic health monitoring of patients on antipsychotic treatment, which ought to lead to early recognition, right management, and, if possible, prevention of metabolic adverse effects.

Key words: antipsychotics, schizophrenia, diabetes, hyperlipidemia

INTRODUCTION

Antipsychotics, both first and second generation, are broad-spectrum neurotherapeutic agents capable of attenuating myriad psychopathological symptoms. The primary indication for antipsychotic drugs is schizophrenia as they are the best treatment now available and they have greatly improved the outlook for individual patients. These medications as first line treatment for schizophrenia are used not only for managing acute phase. Also relapse-preventing efficacy of antipsychotics in the long-term treatment of schizophrenia has been well established. Antipsychotic medications reduce the frequency and intensity of future psychotic episodes in patients who have recovered from an episode. On the contrary, higher relapse rates are seen when medication is discontinued. Except schizophrenia, common conditions with which antipsychotics might be used include schizoaffective disorder, mania, delusional disorder, acute delirium and dementia, together with psychoses associated with a wide range of other diagnoses. They are also effective in controlling the movement disorders associated with Huntington's chorea, Gilles de la Tourette's syndrome, and ballismus and have the ability to treat intractable hiccups and severe nausea and vomiting. Last but not least, antipsychotics are essential to manage qualitative disorders of consciousness associated with various severe somatic disorders.

A range of evidence suggests that treatment with some antipsychotic medications is associated with an increased risk for insulin resistance, hyperglycemia and dyslipidemia compared with no treatment or treatment with alternative antipsychotics (2). On the other hand, several reports suggest, that patients with mental disorders such as schizophrenia

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had an increased prevalence of abnormalities in weight regulation and glucose metabolism prior to initiation of antipsychotic therapy (3).

The aim of this article is to provide a synthesis of the extant literature reporting on the association between antipsychotic usage and glucose and lipid metabolism disturbaces. Raised triglycerides, reduced HDL cholesterol, raised fasting plasma glucose and central (abdominal) obesity are the important componets of the metabolic syndrome. The factor that dominates in obesity is the permanent elevation of plasma free fatty acid and the predominant utilization of lipids by muscles inducing a diminution of glucose uptake and insulin resistance. An insulin-resistant state – as the key phase of metabolic syndrome – constitutes the major risk factor for the development of diabetes mellitus (4). While metabolic syndrome itself is a serious health risk and medical complication, research suggests that this syndrome may place patients at an increased risk for other serious diseases such as coronary heart disease, stroke and myocardial infarction (5, 6). Therefore, it is essential to pay appropriate attention to monitoring metabolic disturbances during antipsychotic therapy, that is, together with our own experiences, also included in the article.

ANTIPSYCHOTICS AND DIABETES

Patients with schizophrenia may be at a higher risk for developing type II diabetes than the general population (7), therefore regular monitoring of glycaemia is essential. The higher prevalence of diabetes among people with schizophrenia could be related to the high prevalence of obesity, as 90% of individuals with type II diabetes are obese (8). Previous studies have provided evidence that some of the second-generation antipsychotics may further increase the risk of type II diabetes. Most of the data in this area consist of case reports of hyperglycemia, diabetic ketoacidosis, and de novo diabetes mellitus (DM) described with multireceptor antagonists (9-15). It´s necessary to mention also lifestyle factors which can certainly contribute to the risk of developing diabetes mellitus in individuals with schizophrenia, with some speculation that genetic factors could also contribute. The negative symptoms of schizophrenia, e.g. loss of social skills, will, drive, and motivation, may be important. Many patients suffer social isolation and reduced activity levels along with greatly increased rates of smoking (16). Food intake in these individuals may be one of the few pleasures in their lives. With regard to their difficult economic situation, they prefer high caloric, often unhealthy foodstuffs. Further compounding the confusion is that much of the literature consists of case reports, poorly controlled studies, or retrospective analyses of populations where the definition of diabetes and/or the means to detect it may be inconsistent or based on older standards.

Le Noury et al. (17) described six of the 394 patients on antipsychotic treatment who developed type II diabetes, 5 males and 1 female. Of these 6, 4 had schizophrenia, one a psychosis linked to prior alcohol abuse and one bipolar disorder. Two of the 6 developed diabetes within 3 years after treatment initiation, both under the age of 45. Two more developed diabetes within 5 years, one under 45 and one over 45. A further two developed diabetes shortly after 5 years of follow-up, one under 45 and one over 45. All 4 patients under the age of 45 were on ongoing treatment with atypical antipsychotics.

It is not known whether antipsychotics exacerbate pre-existing subthreshold DM or disrupt a normal glucose homeostatic system. Weight gain is a robust risk factor for type II DM, therefore, weight gain associated with antipsychotics may be the first step in a cascade of events leading to insulin resistance, glucose intolerance, and DM. However, weight increase is reported in most, but not all, cases. Another explanation leads to serotonin and its receptors which are known to affect glucose homeostasis in a complex, contradictory way. So hyperglycemia with atypical antipsychotics may be due to blockade of 5-HT_{1A} receptors on pancreatic beta cells (18).

Clozapine and olanzapine are the agents most commonly associated with diabetes.

Wirshing and others described development of *de novo* DM in 4 patients receiving clozapine and 2 olanzapine (19). Goldstein and others (20) reported 7 cases of olanzapine-induced hyperglycemia, 2 of which presented with diabetic ketoacidosis. In these patiens, *de novo* DM developed between 5 weeks and 17 months (mean 26 weeks) after treatment initiation. One-half of their sample had known family histories of type II DM, and 4 patients experienced weight gain while taking olanzapine. Other researchers observed patients who were taking clozapine for 5 years and found that as many as 36.6% patients eventually received a diagnosis of type II diabetes (21). Comparison of several medication was made by Lindenmayer and others in a 14-week randomized, double-blind trial. They measured plasma glucose levels in patients receiving clozapine, haloperidol, olanzapine, and risperidone (22). The investigators found significant increases in glucose levels with clozapine and haloperidol at 8 weeks (but not at 14 weeks) and with olanzapine at 14 weeks (but not at 8 weeks). A study that used a large VA (Veterans Affairs) database found significantly higher risks for a diagnosis of diabetes among patiens who were taking clozapine (odds ratio=1.25), olanzapine (odds ratio=1.11), and quetiapine (odds ratio=1.31), but not risperidone (odds ratio=1.05), compared with patients who were taking first-generation antipsychotics (23). The effect was strongest for patients who were younger than 40 years. Also Melkersson and others (24) were comparing conventional antipsychotics and clozapine. Patients receiving clozapine had elevated insulin levels when compared with individuals receiving conventional antipsychotics. In addition, there was a positive correlation between serum concentrations of clozapine and insulin levels. The authors hypothesize that clozapine may result in insulin resistence which subsequently leads to secondary hyperinsulinemia.

However, Wang and others (25) in 2002 contradicted findings of an association between clozapine and diabetes by a study using data from drug benefit programs in New Jersey. In this study, patiens who received clozapine did not have a higher risk of diabetes, compared with patients who did not receive that medication. Surprisingly, chlorpromazine and perphenazine were associated with a significantly higher risk.

Koro and coworkers (26) used large United Kingdom General Practice Research Database with 19 637 individuals with schizophrenia. 451 of them sufferred diabetes whereas patients taking olanzapine had a significantly higher risk of diabetes, compared with those taking first-generation antipsychotics (odds ratio=4.2, 95% CI=1.5–12.2). Risperidone was not associated with a significantly increased risk of diabetes.

Also Zoler (27) tried to assess differential incidence of type II DM associated with antipsychotic treatment. He found out that fluphenazine and risperidone are associated with minimal risk, haloperidol with medium risk, and olanzapine and clozapine with the greatest risk for the development of DM.

The mentioned study results suggest that olanzapine, clozapine and some other antipsychotic drugs may induce insulin resistance, although increased risk for diabetes could also be explained by their liabilities for causing weight gain.

Demographic risk factors for antipsychotic-induced DM have been described. Fourteen of the 15 published case reports by Wirshing and others (19) involved men, and 11 of the 14 cases with noted ethnicity involved people of African descent. It is known that Aboriginals, Hispanics and those of African or Asian descent appear to be at greater risk of developing DM (28). Moreover, many of the reported cases had positive personal or family histories for DM (10-15, 20).

Schizophrenia patients should be evaluated for undiagnosed diabetes by using the criteria recommended by the American Diabetes Association. Factors that indicate a high risk for undiagnosed diabetes include

- a BMI greater than 25
- habitual physical inactivity
- first-degree relative with diabetes
- having delivered a baby heavier than 4 kg (9 lb) or having had gestational diabetes

- hypertension
- an HDL cholesterol level ≤ 0.9 mmol/l (35 mg/dl) and/or a triglyceride level ≥ 6.5 mmol/l (250 mg/dl)
- being a member of a high-risk ethnic population (African American, Hispanic American, Native American, Asian American, Pacific Islander)
- history of abnormal findings on a glucose tolerance test or fasting plasma glucose test
- history of vascular disease (29).

All patients who are starting treatment with antipsychotic agents or whose antipsychotic agent is being changed should be evaluated with a fasting plasma glucose test. If this is not possible, their hemoglobin A1c level should be measured. Fasting glucose levels between 100 mg/dl and 125 mg/dl (from 5.6 to 6.9 mmol/l) are indicative of prediabetes and should prompt closer assessment and follow-up. In case of an abnormal test value (fasting plasma glucose value ≥ 7.0 mmol/l (126 mg/dl), random plasma glucose value > 11.1 mmol/l (200 mg/dl), or hemoglobin A1c value > 6.1%), the next step should be consultation with an internist or other primary health care provider for further assessment because it suggests the possibility of diabetes.

Patients who have significant risk factors for diabetes (BMI ≥ 25, family history, waist circumference ≥ 89 cm for women and ≥ 102 cm for men) should have their fasting plasma glucose level monitored 4 months after starting an antipsychotic and then annualy. Individuals who are gaining weight should be monitored every 4 months. If a patient informs about symptoms of diabetes, a random plasma glucose test should be performed and if the value is elevated (> 7.0 mmol/l if fasting or > 11.1 mmol/l if nonfasting), the patient should be referred to an internist or primary health care provider (30).

ANTIPSYCHOTICS AND HYPERLIPIDEMIA

Certain antipsychotic agents may be associated with hyperlipidemias. It´s known that elevated cholesterol and triglyceride levels participate in the development of coronary heart disease, including myocardial infarction and ischemic heart disease (31, 32). A 10% increase in cholesterol level is associated with a 20%–30% increase in the risk of coronary heart disease and reversely, lowering the cholesterol level by 10% decreases the risk by 20%–30% (33). Also triglyceride levels exceeding 250 mg/dl (2.8 mmol/l) are associated with a twofold higher risk of cardiovascular disease, when compared with lower levels (32, 34).

Wirshing et al. (34) as well as Meyer (35) in their retrospective reports found elevations of lipids in patients who were taking newer antipsychotics. Early case reports, which focused on clozapine found elevated levels of triglycerides but not elevated total cholesterol levels (36, 37, 38). Similarly, results of retrospective study conducted by Osser et al. suggest that olanzapine has significant effects on both weight and serum triglyceride levels, while fasting total cholesterol does not increase. After 12 weeks of olanzapine treatment, the group mean body weight increased 5.4 kg (11.9 lb) and the mean triglyceride levels rose by 37%. In addition, there was a strong association between weight change and triglyceride change (39).

Meyer compared lipid changes between risperidone- and olanzapine-treated patients after one year. Olanzapine therapy was associated with significantly greater increases in triglycerides and cholesterol levels than risperidone, but in this case the increases were not correlated with changes in weight parameters (40). Another study that used the United Kingdom General Practice Research Database (41) included 18 309 individuals with schizophrenia. Patients who received olanzapine had significantly increased odds of developing hyperlipidemia, compared to patients who received no antipsychotic (odds ratio=4.65, $p<0.0001$) and compared to patients who received a first-generation antipsychotic (odds

ratio=3.36, p=0.0002). In contrast, risperidone was not associated with an increased risk in either comparison.

Rettenbacher and others (42) conducted in 2007 a prospective, open study, in order to compare serum lipids during treatment with amisulpride, ziprasidone, clozapine and olanzapine over a period of 4 weeks. Total cholesterol and triglycerides increased in patients treated with clozapine and olanzapine whereas high-density lipoprotein cholesterol decreased in those patients. Amisulpride and ziprasidone treatment lead to decrease in total cholesterol whereas high-density lipoprotein cholesterol increased. This ziprasidone´s significant favourable effects on total cholesterol, LDL, and HDL were also observed by Brown and Estoup (43).

In summary, the treatment with ziprasidone and amisulpride appears to be more favourable than treatment with clozapine and olanzapine with respect to the risk to induce weight gain and hyperlipidaemia. However, considering individuals with schizophrenia are at a higher risk for atherosclerotic heart disease, their lipid profiles should be regularly monitored according to guidelines established by the National Cholesterol Education Program and the U.S. Preventive Services Task Force for patients at increased risk for coronary heart disease (44). Psychiatrists should be aware of the lipid profile of each patient with schizophrenia they treat. The lipid panel should include measurements of total cholesterol, low density lipoprotein (LDL) as well as HDL cholesterol, and triglyceride levels. Lipid screening should be carried out at least once every 2 years when the LDL level is normal and once every 6 months when the LDL level is greater than 3.36 mmol/l (130 mg/dl). When patients with abnormal levels are identified (for example, LDL level exceeding 3.36 mmol/l), the patient should be referred to a specialist, or eventually, change of medication should be considered in serious cases (30).

We also have experiences with metabolic disturbances associated with antipsychotic treatment. In the group of one hundred patients receiving antipsychotic medication, hyperglycaemia was found in 20% of the patients. In two of them was also diagnosed new onset diabetes mellitus during the hospitalization. These results were similar to those of Le Noury et al. (2008) which were mentioned before. Hypertriacylglycerolemia was observed in 29 percent of patients, more of them (46%) experienced elevated serum total cholesterol levels. In contrast with findings of several authors, we have found increase not only in triacylglycerol levels but also in total cholesterol levels.

The results confirm increased prevalence of abnormalities in glucose and lipid metabolism associated with antipsychotic administration. However, a ***genetic*** background as well as ***unhealthy dietary habits***, smoking and lack of physical activity may be possibile risk ***factors*** contributing to development of these disturbances.

CONCLUSIONS

Data from various sources on the use of atypical antipsychotics indicate that some drugs in this class are associated with a significant risk for weight gain, dyslipidemia and disordered glucose metabolism, which are the components of metabolic syndrome, an established cardiovascular risk factor. Adverse effects on glucose metabolism have more frequently and consistently been associated with clozapine and olanzapine treatment with discrepant reports for quetiapine and risperidone (45). Regarding serum lipids, the structurally related dibenzodiazepine-derived atypical antipsychotics such as clozapine, olanzapine and quetiapine are associated with greater elevations in serum triglycerides than in total cholesterol, whereas the nondibenzodiazepine agents including risperidone, ziprasidone and aripiprazol have minimal effects on lipids. These observations raise concerns about the potential differential long-term deleterious effects of some antipsychotics on cardiovascular health.

We cannot rely only on weight gain as the clinical marker that defines individuals who may have underlying metabolic disturbances. Notably, increase in body weight is not an absolute prerequisite for the development of insulin resistance, impaired glucose tolarance or type II diabetes mellitus during antipsychotic treatment, as reflected by the subgroup of patients who present with ketoacidosis but no significant weight gain. The increased risk of dyslipidemia or diabetes is not just a product of weight gain, that is why there is an urgent clinical need to *monitor every antipsychotic*-treated patient for *metabolic disturbances.* Certainly, lifestyle factors, particularly poor dietary habits, inactivity, smoking and the use of alcohol, may also contribute to development of these abnormalities.

This article provides recommendations for the systematic health monitoring of individuals with schizophrenia and other mental disorders for whom antipsychotic medication is prescribed, regarding which health parameters and when they should be monitored. Subsequently, this physical health monitoring ought to lead to early recognition, right management, and, if possible, prevention of metabolic adverse reactions in patients taking an antipsychotic medication. Hopefully, it could also prevent from metabolic morbidity and lower the prevalence of cardiovascular disease – a leading cause of death in persons with mental disorders ad schizoprenia (46). Published monitoring protocols for patiens prescribed atypical antipsychotics are presented in table 1 (47).

Despite the evidence that psychiatric patients are at high risk for metabolic problems and poor health outcomes secondary to complications from diabetes and cardiovascular disease, this is not true for all patients. Certainly, they can not be deprived of efficacious agents out of fear of metabolic complications, especially when a number of patients do not carry this risk.

Tab. 1. Published monitoring protocols for patients prescribed atypical antipsychotics

Metabolic parameter	weight	waist circumference	blood pressure	fasting plasma glucose	glycosylated hemoglobin test	fasting lipid profile
baseline	X[1,2]	X[1,3]	X[1,3]	X[1,2,3,4]	X[2,4]	X[1,3]
each visit for 6 months	X[2]					
each visit for 4 weeks	X[1]			X[3]		
each visit for 8 weeks	X[1]					
each visit for 12 weeks	X[1,3]	X[3]	X[1]	X[1]		X[1]
each visit for 4 months				X[2,4]	X[2,4]	
quarterly	X[1,2]					
every 6 months			X[3]	X[3]		X[2,3]
annually		X[1]	X[1]	X[1,2,4]	X[2]	
every 2 years						X[2]
every 5 years						X[1]

[1]American Diabetes Association et al. 2004 (45)
[2]Marder et al. 2004 (30)
[3]Lambert et al. 2004 (48)
[4]Expert Group 2004 (49)

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ANTITUSSIVE ACTIVE HERBAL POLYSACCHARIDES ISOLATED FROM MALIAN MEDICINAL PLANTS TRICHILIA EMETICA VAHL. (MELIACEAE), AND OPILIA CELTIDIFOLIA GUILL. & PERR. ENDL. EX WALP. (OPILIACEAE)

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Abstract

Several therapeutic effects have been described for traditional medicinal plants in Mali, *Trichilia emetica* Vahl. (Meliaceae), and *Opilia celtidifolia* Guill. & Perr. Endl. ex Walp. (Opiliaceae). These herbs contain a typically high carbohydrate compounds proportion, especially those polysaccharides, e.g. arabinogalactan and rhamnogalacturonan, which antitussive effect was verified in experiments.

The water and water-ethanol soluble polysaccharide materials were isolated from the leaves of *Trichilia emetica* and *Opilia celtidifolia*, structurally characterized and introduced in tests of antitussive activity and airways smooth muscle reactivity in conscious guinea pigs model. The cough reflex was induced by citric acid aerosol and intensity of cough response was expressed as number of cough efforts. The airways smooth muscle reactivity was exposed as values of airways specific resistance calculated according to Pennock in conditions *in vivo*.

T. emetica and *O. celtidifolia* polysaccharides possessed significant cough-suppressive effect on citric acid-induced number of cough efforts. Furthermore, changes of specific airways resistance values referred to bronchodilatory property of polysaccharides isolated from *O. celtidifolia*.

These results provide a support to the claims by the traditional medicine practitioners about the usefulness of the leaves of *T. emetica* and *O. celtidifolia* in the treatment of respiratory diseases accompanied with cough and bronchoconstriction.

Key words: antitussive activity, cough, polysaccharides, *Trichilia emetica*, *Opilia celtidifolia*, codeine

INTRODUCTION

In the traditional medicine plants have been used to treat various types of illness as well as wounds healing, both external and internal. Herbal polysaccharides have been the subject of studies for a very long time, mainly focused on their physical properties, chemical and physical modification and application. Over the last years there has been increasing interest in the biological activity of the natural polysaccharide polymers (1, 2). The present day researches aim one's effort to identification of the active substances responsible for therapeutic effects. Some such polysaccharides have been developed into drugs and show clinical efficacy and, moreover, the mentioned polysaccharides are almost non-toxic (3).

Many authors confirmed antitussive ability of herbal polysaccharide complexes contained considerable high proportion of arabinogalactan and rhamnogalacturonan, which were identified and isolated from *Althaea officinalis, Malva mauritiana, Arctium lappa* and *Salvia officinalis* (4-8). Presented study was focused on potential effect on defense airways reflexes of the popular Malian medicinal plants *Trichilia emetica* and *Opilia celtidifolia,* the typical mucilaginous plants, from which various polysaccharides were isolated. The result of chemical analysis of these leaves polysaccharide complexes showed that contained high proportion of both arabinogalactan (*Trichilia emetic*a 54% and *Opilia celtidifolia* 60%) and rhamnogalacturonan (*Trichilia emetic*a 15% and *Opilia celtidifolia* 30%) (9, 10).

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Trichilia emetica Vahl (*Meliaceae*) is a popular medicinal plant in poor country located in West Africa – Mali. Furthermore, it grows naturally through sub-Saharan Africa from Senegal to the Red Sea, throughout East and Central Africa to Congo and South Africa. This evergreen plant is called by indigenous population "Soulafinzan". It is commonly used in folk medicine for the treatment of various diseases (11). Powder of the grounded roots is used against cirrhosis, onchocerciasis, ascariasis, stomach-aches and dysmenorrhoea. This powder mixed with milk is used as a purgative and poison antidote, and mixed with honey, it is used against asthma. The powder mixed with lukewarm water is used for vomiting. Stem barks powder is traditionally used against fever, cough and bronchial trouble. Furthermore, the bark is associated with gastritis, hepatitis, internal tumors, and ulcers treatment. The leaves are used in decoctions against various diseases, e.g. malaria, hypertension, lumbago and wounds (12).

Opilia celtidifolia Guill. & Perr. Endl. ex Walp. (*Opiliaceae*) is a woody climber, spreading, which grows in fringing forest and savannah, often on anthills. It is widespread from Senegal to Nigeria and dispersed over the drier parts of tropical Africa (13). *Opilia celtidifolia* is well known to the traditional healers as a remedy to cure several diseases, mostly dermatitis and malaria. Decoction of the leaves is used as febrifuge, antitussive, gargle, against dental abscesses, acting as a purgative. A macerate is particularly effective in expelling worms from children (14).

Recently, there were reports that both plant polysaccharide complexes exhibited complement-fixing and macrophages-stimulating activities (12). Furthermore, *Opilia celtidifolia* polysaccharides possessed influence on nitric oxide metabolism *in vitro* conditions (14). Plant polysaccharides with strong anti-inflammatory ability had shown significant cough suppression in our previous experiments (8). A large scale of reported medicinal effects of Malian plants *T. emetica* and *O. celtidifolia,* structural constituents of polysaccharide complexes as well as the finding that some of these polysaccharides showed significant biological activities prompted us to verify experimentally their possible antitussive effect and impact on the other airways defense reflexes.

MATERIAL AND METHODS

Animals

Adult male Trik strain guinea-pigs, weighing 200 – 350 g were supplied by the Department of Experimental pharmacology, Slovak Academy of Science, Dobra Voda, Slovakia, kept in the animal house with food and water *ad libidum* and with a standard air conditioning system. The animals kept one week in quarantine before starting the experiment.

Each of polysaccharides as well as both control agents ("positive" codeine, "negative" vehicle) were tested on individual group of animals consisting of 8 guinea pigs. Total number of animals used in the study was 32.

The experimental protocols were approved by the institutional Ethics Committee of the Jessenius Faculty of Medicine, Comenius University in Martin, Slovakia and complied with Slovakian and European Community regulations for use of laboratory animals.

Plant material

The leaves of medicinal plants *Opilia celtidifolia* and *Trichilia emetica* were purchased from Bamako market, Mali, West Africa. Voucher specimens are deposited at the herbarium of the Department of Traditional Medicine, Mali, West Africa.

Both plants leaves were air dried and pulverized to fine powder. Then *O. celtidifolia* powder was pre-extracted under reflux with chloroform, macerated in 80% ethanol followed by methanol to remove lipophilic and colored extractive compounds. Powder of *T. emetica* was extracted with 80% ethanol under reflux for 5 h and filtered. The drug residues were further extracted twice with distilled water at 50 °C and 100 °C, filtered, and freeze-dried to

give crude polysaccharide fractions OC 50, TE 50 and OC 100, TE 100. Fractions OC 100 and TE 100 were chosen for further biological tests.

Experimental procedure

Awaken guinea-pigs were individually placed in a bodyplethysmograph box (HSE type 855, Hugo Sachs Elektronik, Germany) and restricted so that the head protrudes into the nasal chamber and the neck were sealed with a soft diaphragm. The cough was induced by citric acid in a concentration 0.3 M. The citric acid aerosol was generated by a jet nebulizer (PARI jet nebulizer, Paul Ritzau, Pari-Werk, Germany, output 5 $l.s^{-1}$, particles mass median diameter 1,2 μm) and delivered to the head chamber of the plethysmograph for 3 min interval. The intensity of cough response was defined as **number of cough efforts** counted in mentioned time interval. The cough effort was defined as sudden PC-recorded enhancement of expiratory flow associated with typical cough motion and sound followed by a trained observer.

The reactivity of the airway smooth muscle *in vivo* conditions was expressed as values of **specific airway resistance** calculated according Pennock et al. (15) by time difference between pressure changes in head and chest parts of bodyplethysmograph during normal breathing pattern.

The influence of polysaccharides and control drugs (codeine and vehicle) both on citric acid-induced number of cough efforts and values of specific airway resistance, were registered **before** any agent application (values labeled as N in graphs) and after that in **60 min time interval.** According to our previous experiments almost best cough suppression after orally applied plant polysaccharides was recorded in this selected time interval measurement (4, 5, 8)

All used substances were applied by oral route of administration. The plant polysaccharides were dissolved in water for injection (50 mg of polysaccharide substance in 2 ml of vehicle) and were administered in the dose of 50 $mg.kg^{-1}$ b. w. Positive control agent codeine was dissolved in the same vehicle and applied in the dose 10 $mg.kg^{-1}$ b. w. Vehicle was tested under the same conditions in the dose 1 $ml.kg^{-1}$ b. w.

Statistics

The changes of number of the citric acid-induced cough efforts and values of specific airways resistance in 60 min interval were compared and statistically evaluated with initial data (N). Student-t test was used for the statistical analyses of the obtained results. Data are presented as mean ± standard error of the mean (S.E.M.). $P< 0.05$ was considered statistically significant. Significance of $p< 0.05$ and $p< 0.01$ is shown by one and two asterisks, respectively.

RESULTS

From *T. emetica* (TE) leaves was extracted polysaccharide material rich mainly in galactose (~29%) and arabinose (~25%), and galacturonic acid (~21%). It indicates to the presence of arabinogalactan type of polymer (~54%) associated with rhamnogalacturonan (~30%) (1).

Compositional analysis of *O. celtidifolia* (OC) polysaccharide revealed the dominance of galactose (~32%) and arabinose (~27%) residues indicated the presence of arabinogalactan (~60%) and a rhamnogalacturonan (~14%) or their complex.

By the chemical induction of cough reflex a significant reduction of the number of coughs efforts (NE) 60 min (60 vs. N, $p< 0.05$) after oral administration of *T. emetica* polysaccharides in the dose of 50 $mg.kg^{-1}$ b. w. were observed. Similarly, oral administration of *O. celtidifolia* polysaccharides in the dose of 50 $mg.kg^{-1}$ b. w. significantly decreased a number of cough efforts in 60 min time interval (60 vs. N, $p < 0.05$) after administration. Our results

showed that the suppression of number of the cough efforts after application polysaccharides from *T. emetica* and *O. celtidifolia* was lower in comparison with efficacy of codeine but the differences were not found statistically significant (Fig. 1). Vehicle almost did not change initial number of cough efforts (N= 6.37; 60= 6.12).

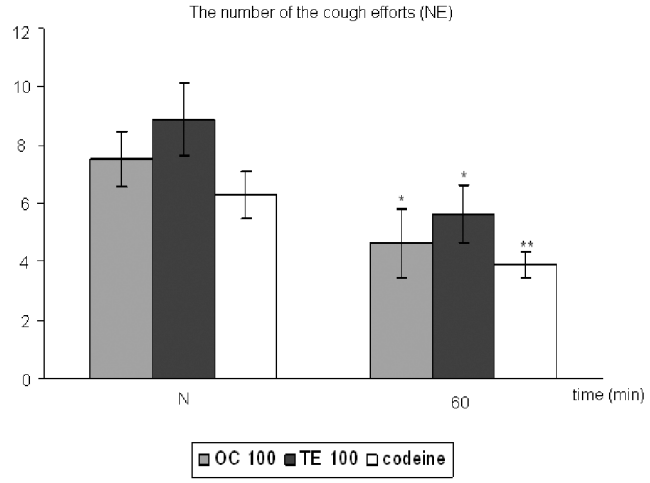

Fig. 1. The influence of polysaccharides from *O. celtidifolia* (OC 100) and *T. emetica* (TE 100) and codeine on the citric acid-induced cough efforts (NE) in guinea-pigs recorded 60 min after orally administered polysaccharides (dose 50 mg.kg^{-1} b.w.) and control agent (dose of codeine 10 mg.kg^{-1} b.w.). ** p< 0.01; * p< 0.05; N – Initial values before application of the polysaccharides and codeine

Tab.1. Results – The changes of specific airway resistance (R.V) *in vivo* conditions after *T. emetica*, *O. celtidifolia* and codeine oral administration;**p< 0.01; S.E.M. – standard error of means

	N (mean ± S.E.M.)	60 (mean ± S.E.M.)	Significance (N vs. 60)
Trichilia emetica	6.29 ± 0.67	6.11 ± 0.64	
Opilia celtidifolia	7.13 ± 0.63	3.59 ± 0.62	**
Codeine	9.48 ± 1.04	9.53 ± 0.60	

As shown in Table 1, polysaccharides isolated from *O. celtidifolia* significantly decreased the values of specific airway resistance measured under the same conditions (60 vs. N, p< 0.01), which pointed at bronchodilatory potency of polysaccharide sample. Contrariwise, polysaccharides of *T. emetica* as well as codeine did not influence the values of specific airway resistance after 60 min.

DISCUSSION

The coughing is normal physiological response to an irritation of the laryngo-tracheobronchial system caused by mechanical or chemical stimulation (16), but it is also common symptom of many respiratory and non-respiratory disorders (17, 18). When cough becomes

problematic, as when it is mediated by disease, it can causes distress, affects sleep, rest, social activity and hence interfere significantly with quality of life. In this case it is necessary to suppress the cough or maintain it at tolerable grade (19). An ideal drug would reduce the increased sensitivity of the reflex to normal either by altering the disease process or by reducing the response of the sensory nerves in the lung (20).

The antitussives from the group of narcotic analgesics, so-called "codeine group" are widely used in clinical conditions. Their cough-suppressive action is very strong in doses below those required for pain relief. Although excellently efficacious, they are associated with relatively high rate of unwanted effects, like depression of the respiratory centre, decreased mucus secretion in bronchioles, as well as inhibition of ciliary activity (21). Furthermore, their administration increases sputum viscosity, decreases expectoration, induces hypotension and constipation, which limits their clinical use. All of these facts urge to look for other non-narcotic substances preventing the pathological cough (22). Many proprietary cough preparations contain mainly saccharides. They have been shown to reduce cough in normal individuals in response to challenge with citric acid and capsaicin for a short time (20).

In the present study the antitussive effects of polysaccharide extracts from *Trichilia emetica* and *Opilia celtidifolia* were evaluated. The results of the present study demonstrated that polysaccharides isolated from the leaves of *Trichilia emetica* and *Opilia celtidifolia* possess significant antitussive activity on citric acid-induced cough efforts. Both polysaccharides samples contained as main structural compound arabinogalactan and lower proportion of rhamnogalacturonan, the polysaccharides with experimentally confirmed antitussive property in conscious cats as well as guinea pigs testing systems (4, 5, 23). They could be constituents responsible for cough suppression observed after *Trichilia emetica* and *Opilia celtidifolia* leaves polysaccharides administration.

Despite extensive research, certain mechanisms of polysaccharides antitussives are still poorly understood. However, orally applied and swallowed polysaccharides don't reach airways and they are not absorbed from the gastrointestinal mucosa because of high molecular size, there are various hypotheses explaining their cough-suppressive ability:

a) During the swallowing act are polysaccharides in direct contact with pharyngo-epipharyngeal mucous area, in which nerve terminals regulating cough are located. Previously, Mazzone et al. (24) demonstrated that these structures stimulated increased cough receptors sensitivity and vice versa inhibition reduces cough response. These findings confirmed by Irwin and Madison (25), which had reported the efficacy of nasal antihistamines and corticoids in allergic rhinitis as results of epipharyngeal neurons inhibition. We could speculate if polysaccharides modulate neuronal activity, or if they are only covering mucosa in this area and this way preventing contact of nerve terminals with irritants (26, 27).

b) Other theory regards their mucoactive attributes. Increased production of the thin phlegm could be induced through certain reflexive mechanism, e.g. vago-vagal. It is generally accepted that interaction of agents with vagal nerve endings in gastric mucosa influencing heart activity and contributes to regulation of airways smooth muscle tone and function of serous bronchial glands (25). Previously, it was shown that herbs-originated saponins, e.g. from mullein, which are very antitussive active, non-absorbing substances, structurally similar to polysaccharides, acted in airways via gastro-pulmonary vagal reflex (28).

c) Another important carbohydrates property includes increasing hypersalivation, and swallowing in consequence this activity. It was found that increased saliva production and swallowing interferes with the cough reflex and lead to cough suppression (29).

d) Observed cough suppression could be accompanied with bronchodilatory effect of polysaccharides. Previously, it was reported that bronchoconstrictors were able to provoke cough reflex (30) and agents with strong bronchodilatory activity were also able to suppress the coughing (31). We recorded strong inhibition of citric acid-induced bronchoconstriction as result of orally applied *O. celtidifolia* polysaccharides, which supported our supposition about bronchodilation participated in achieving cough-suppressive effect.

CONCLUSION

Summarizing the results of the present study we can report that herbal polysaccharides isolated from the leaves of *Trichilia emetica* and *Opilia celtidifolia* possess antitussive activity on citric acid-induced cough. Furthermore, *Opilia celtidifolia* carbohydrates attenuated citric acid-induced bronchoconstriction, which could participate in registered cough suppression. These results support traditional use of both plants for therapy of respiratory diseases associated with cough and airways hyperreactivity.

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ARCHITECTONIC CHANGES OF THE SUBPAPILLARY VASCULAR PLEXUS IN THE PAPILLARY PART OF THE DERMIS WITHIN AGEING HUMAN SKIN

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Abstract

Available studies confirm the decreased capillary density within the papillary dermis in ageing skin. This study was aimed at the observation of the architectonic changes of the subpapillary vascular plexus especially capillaries during reduction of the capillary network in the papillary dermis. Skin specimens (75) were taken from the anterior thoracic region of the cadavers in age range 34 – 82 years. Samples were fixed in formalin and embedded into the parafin blocks. CD34 immunohistochemical method was used for the detection of the capillary endothelium. The shape of the capillary loops and their mutual arrangement in the papillary line were studied in parallel and perpendicular sections. Image analyser Vision Assistant version 7.1.1 was used for the exact evaluation of the architectonic parameters. Authors cannot confirm the individual variability of the architectonic arrangement of the capillary network in the papillary line inside age groups. However, statistical analysis between various age groups confirm statistically significant difference as for the parameter of intercapillary distance between the groups of 63 - 64 and 71 – 74 years old individuals. Results of the study showed that decreased capillary density before the 8th decennium is probably caused mainly by the shortening of the capillary loops, however, another decrease of the capillary density during the 8th decennium is probably caused mainly by the decreased number of the capillary loops in the the papillary dermis.

Key words: skin, ageing, capillary, subpapillary plexus, architecture

INTRODUCTION

Blood supply of the skin is formed by two plexuses: superficial subpapillary plexus and deep reticular plexus, both situated within the dermis. Superficial subpapillary plexus forms the microcirculatory bed in the papillary dermis and the upper part of reticular dermis, lower reticular dermis and hypodermis take nutritional support from the deep reticular plexus (1, 2, 3).

Superficial subpapillary plexus is much more influenced by the processes of intrinsic and extrinsic ageing. It is formed by the segments of terminal arterioles, capillaries and postcapillary venules 1 - 1.5 mm below the skin surface (4, 5). Terminal arterioles have a function of the praecapillary sphicter, postcapillary venules are physiologically the most reactive segment of the subpapillary microcirculation. Capillaries form subepidermal capillary loops - in each dermal papilla there is the only one capillary loop (6). There are approximately 40 capillary loops per mm^2 (7).

Available studies rewiev characteristic qualitative and quantitative microvascular changes caused by biological ageing and photoageing. Electron microscopic observations reveal the ultrastructural changes of the superficial vascular plexus to the intent of the appearance of the thinwalled vessels and endothelial cells irregularities (5, 6, 11). Light microscopic and videocapillaroscopic quantitative studies present the decreased capillary density within the papillary dermis in elderly (8, 9, 10).

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Our study is aimed at the observation of the architectonic changes of the subpapillary vascular plexus especially capillaries which accompany decreased capillary density of the papillary dermis in ageing skin.

MATERIAL AND METHODS

Skin specimens (75) were taken from the cadavers given for the anatomical dissections in age range 34 – 82 years. Skin samples were excised from the anterior thoracic regions, fixed in formalin and embedded into the parraffin blocks. Serial parallel and perpendicular sections were made. Paraffin sections were stained by hematoxyline eosine and processed by CD34 immunohistochemical method (M 7165 CD34 Class clone QBE end 10 DAKO Cytomation) for the detection of the capillary endothelium. The shape and mutual positions of the capillary loops of the superficial subpapillary plexus were studied in various age groups in perpendicular sections and parallel sections (approximately in the papillary line). The comparison of the architecture of the capillary network in the papillary line in various age groups was made according to two parameters – intercapillary distance and a number

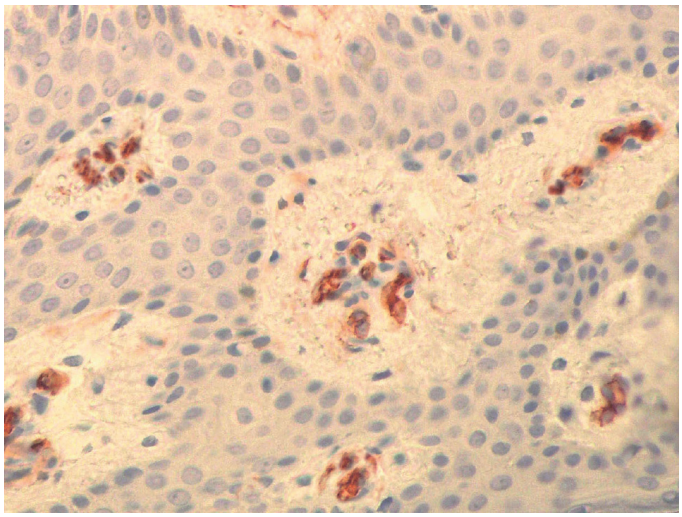

Fig. 1. Parallel section of the skin from the anterior thoracic region of 36 years old woman. CD34 immunohistochemical method, magnification x 200.

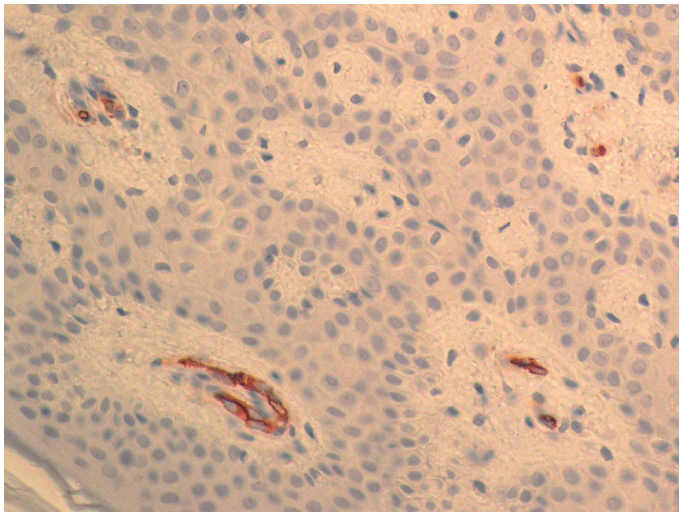

Fig. 2. Parallel section of the skin from the anterior thoracic region of 73 years old woman. CD34 immunohistochemical method, magnification x 200.

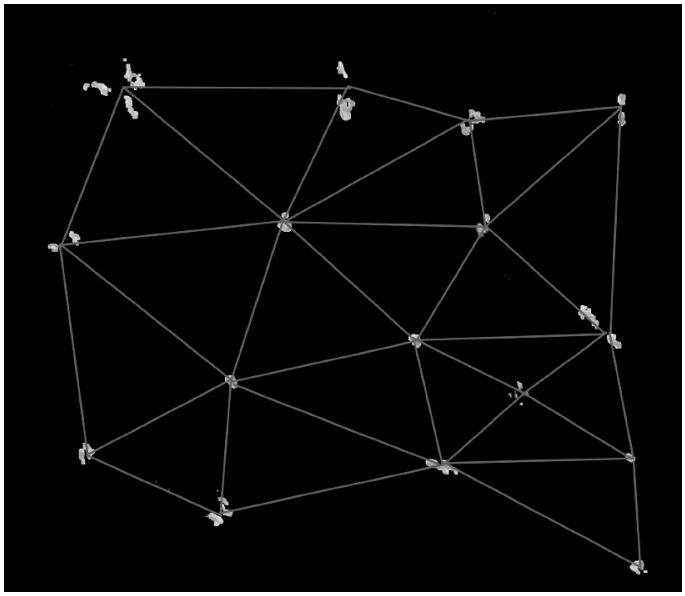

Fig. 3. Binary map made by Vision Assistant with Delaunay triangulation –biopsy specimen taken from the anterior thoracic region of 43 years old man - parallel section.

of adjacent capillary loops. The microscopic views in constant magnification (x120) were photographed by the digital camera system (Olympus Camedia system 4.0 megapixel installed in Nikon EPI-FL3 13414 Japan). Digital pictures were processed into the binary maps with Delaunay triangulation using Vision Assistant software version 7.1.1. Delaunay triangulation of the points - marking the axies of the capillary loops was made for the best determination of assigned parameters. The intercapillary distance was determined as a distance between two adjacent axies of the capillary loops. These parameters were measured between all neighbouring capillary loops in Delaunay triangulation. The intercapillary distance was measured in units specified in preferences. All obtained values of intercapillary distances and a number of adjacent capillaries were statistically processed by ANOVA test and t–test in Microsoft Excel.

RESULTS

The objective of the study was the observation of architectonic changes of the capillary network within the papillary dermis during diminution of the capillary density. Accessible studies showed significantly higher capillary density in young individuals in comparison to the elderly persons.

The shape of the capillary loops observed in perpendicular sections showed a variability as for the height and the course in various age groups. There were high and wavy capillary loops in the skin of younger persons. With aging the capillary loops became lower and copied flattened dermoepidermal junction. Determination of mutual arrangement of the capillary loops in the papillary line was done due to the intercapillary distance and a number of adjacent capillaries. Statistical analysis does not confirm significant individual differences in the age groups. It means that we cannot confirm the individual variability of the geometrical arrangement of the capillary network in the papillary line in the same age. However statistical analysis between various age groups confirm statistically significant difference as for the parameter of intercapillary distance between the groups of 63 - 64 and 71 – 74 years old individuals. According to this result we can presume that the number of capillaries per area of the papillary dermis is reduced in the skin taken from 71 – 74 years old individuals compared to the younger individuals.

Tab. 1. Average values of the intercapillary distances in the papillary line.

Age'	Intercapillary distance (units)		ANOVA test inside the age group p value	T test Comparison p value
	average	sd		
34-36''	162.91	21.36	0.282	
42-44	166.58	18.42	0.478	0.883
54-55	163.78	20.10	0.857	0.892
63-64	170.14	17.18	0.696	0.876
71-74	194.54	16.44	0.979	**0.047**
80-82	195.72	18.80	0.322	

Tab. 2. Average values of the number of adjacent capillary loops in the papillary line.

Age'	Number of adjacent capillaries		ANOVA test inside the age group	ANOVA test all age groups p value
	average	sd		
34-36	5.43	0.69	0.323	
42-44	5.53	0.99	0.586	
54-55	5.43	1.04	0.899	0.646
63-64	5.43	1.08	0.827	
71-74	5.33	0.79	0.816	
80-82	5.14	1.03	0.626	

Another observed parameter was the number of the nearest adjacent capillary loops. No statistically significant differences in comparison of this parameter were determined, therefore we could state that the mutual horizontal geometrical organization of the capillaries within the papillary line is similar but due to the different magnification because of the decreased intercapillary distance – less capillary loops per area in ageing skin.

On the basis of the statistical analysis of the established parameters we can suggest that decreased capillary density before the 8th decennium is caused by the changed shape of the capillary loops - its height in the papillae, but during the 8th decennium decreased capillary density is probably caused also by the decreased number of the capillary loops in the papillary dermis.

DISCUSSION AND CONCLUSIONS

The study was aimed on the observation of the organization of the capillary network of the subpapillary plexus because of its important physiological functions - nutritive and thermoregulatory, but also in skin reactivity during pathological conditions. All these functions can be influenced by the the microvascular changes in ageing skin.

Electron microscopic studies of the microcirculation in ageing skin revealed unspecific

irregularities of the endothelial cells and an appearance of the thinwalled vessels in the papillary dermis caused by the decreased metabolic activity of the veil cells (4, 5).

Reduction of the capillary network within ageing skin is reviewed by majority of the authors (8, 9, 10). Results of our study contribute that that decreased capillary density before the 8th decennium is caused by the changed shape of the capillary loops - its height in the papillae, but during the 8th decennium decreased capillary density is caused also by the decreased number of the capillary loops in the papillary dermis. The dissapearance of these vessels and concurrent thining of the epidermis results in exposure of the larger vessels of the subpapillary plexus which then tends to become dilated (8, 9). Wider horizontal subpapillary plexus, the lack of the capillary loops and more branching in the blocks of the older individuals were described also by Richardson et al. (9). Gilchrest et al. (10) detected decreased number of the vertical vessels of the superficial subpapillary plexus in buttock skin of the older subjects which is probably caused by flalttening of the capillary loops in low dermal papillae .

On the other hand, in our study of the organization of the subpapillary plexus in various body regions we found out that the different capillary density in various body region in one age range was caused only by the different shape – lenght of the capillary loop, not by the number of the capillaries(12). Similar supposition was described by Miniati et al. (13), Santhilier et al. (14, 15) and Hern et Mortimer (16).

Detailed knowledge of microcirculation in ageing skin is important for the basic morphological understanding of the changed physiologic functions in elderly.

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OCCUPATIONAL EXPOSURE TO VOLATILE ANAESTHETICS, CHROMOSOMAL ABERRATIONS AND NUCLEOTIDE EXCISION REPAIR POLYMORPHISMS OF GENES *XPD, XPG, XPC*

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Abstract

Authors evaluated the incidence of total chromosomal aberrations (CA) and their types – chromatid-type (CTA) and chromosome-type (CSA) in peripheral blood lymphocytes from 76 anaesthesiologic workers occupationally exposed to volatile anaesthetics in relationship to polymorphisms of DNA repair genes *XPD*, *XPG* and *XPC*. The cytogenetic analysis was used for determination of chromosomal aberrations frequency and PCR-RFLP method for polymorphisms of genes. Statistically higher incidence of total CAs was detected in exposed group as compared to control (2.53±1.46% vs. 1.26±0.93%; Mann-Whitney U-test; P=0.0008). There was detected statistically higher incidence of CSA-type of aberrations in comparison to CTA-type (1,92±1,38% vs. 0,61±0,83%; Mann-Whitney U-test; P=0,0009). Similarly, it was detected both the increase of total CAs and CTA-type and CSA-type in presence of variant allele in genes *XPD* exon 23 and *XPC* exon 15. The differences were not statistically significant. It wasn´t detected any difference of total CAs, CTA-type and CSA-type in gene *XPG* exon 15 with presence of variant allele C. Authors pointed out the importance of individual susceptibility factors in evaluation of effects of genotoxic agents.

Key words: volatile anaesthetics, chromosomal aberrations, occupational exposure, DNA-repair genes *XPD, XPG, XPC*.

INTRODUCTION

The anaesthesiologists are the unseparable part of operation teams of theatres and they are excessively occupationally exposed to volatile anaesthetics. The mutagenic and carcinogenic effect of them has been permanently discussed [1, 2]. Halothane is used minimally and often substituted by other with less toxic anaesthetics as: Sevoflurane, Isoflurane, Desflurane, Entrane. These anaesthetics are all halogenous aliphatic hydrocarbons. The genotoxicity of volatile anaesthetics halothane and isoflurane were assessed *in vitro* in human lymphocytes. The peripheral blood lymphocytes exposed to 1mM isoflurane were capable the complete reparations during 60 minutes, whereas cells exposed 0.1mM halothane only partially after 120 minutes [3]. Genotoxicity of desflurane was assessed also by comet assay as extent of DNA fragmentation in peripheral human lymphocytes *in vitro*. There was detected the increased DNA migration not only in cells exposed halothane, but also in cells exposed to desflurane [4]. After short-term exposure to sevoflurane did not arise the induction of sister chromatid exchanges in peripheral lymphocytes [5]. Anaesthetics caused irritation of airways of volunteers. The observed extent of the damage was the highest in sevoflurane and was decreased in following order: halothane, isoflurane and desflurane. No increase in the number of chromosomal aberrations was detected in patients exposed to anaesthetics during operation evidencing, that the long term or repetitive exposure to anaesthetics can caused genetic changes only [6]. Prokes evaluated the

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halothane vapour in theatres and detected, that the most exposed group within anaesthesiologists are nurses [7]. The frequency of sister chromatid exchanges in peripheral blood lymphocytes was assessed by Hoerauf et al. [8] in a group of medical workers exposed to nitrous oxide and isoflurane. They were exposed to 11.8 ppm nitrous oxide and 0.5 ppm isoflurane. The mentioned exposure caused increase in frequency of SCE (average 9.0). Exposure to residues of anaesthetics can increase the genetic damage comparable with the damage caused by smoking (11-20 cigarettes per day) [8]. Bozkurt et al. [9] confirmed no relationship between occupational exposure to volatile anaesthetics and increased frequency of SCE in peripheral blood lymphocytes. The increased incidence of CA was detected by Rozgaj et al. in group of 129 medical workers in operating rooms [10]. There was not detected any dependence on job categorization, however, operation physicians showed increased frequency of dicentric chromosomes. Rozgaj and Kasuba [11] and Rozgaj et al. [12] assessed frequencies of CA, MN and SCE in anaesthesiologists, and found them increased particularly in females. Bilban et al. [13] studied frequencies of CAs, SCE and MN in medical workers of operating and reanimation rooms. They found out increased frequency of CAs (2.69%), which was comparable to a group of radiologists. The increase of DNA damages was detected as well by Chandrasekhar et al. [14] using comet assay, MN test and peripheral blood lymphocytes analysis in buccal mucous cells. However, their findings were not dependent on age and gender of observed persons.

MATERIAL AND METHODS

The study was concluded on 152 exposed and control individuals and the frequency of chromosomal aberrations and polymorphisms of DNA repair genes was analysed. All of examined persons completed history questionnaire about length and way of exposure, job category, exogenous factors (smoke, drug usage, exposure to radiation, alcohol consumption and dietary) before blood collection and give an agreement to be involved in the study.

Exposed group consists of 76 workers from Faculty Hospital in Martin (n = 60) and Central Military Hospital in Ružomberok (n = 16). All of these workers were regularly exposed to volatile anaesthetics. By job grade they are classified as anaesthesiologic physicians and nurses. They participated in application of volatile anaesthetics during surgical interventions in complete anaesthesia in operating rooms. There were predominantly female persons (N = 61). Smokers (23) form 30.26% and physicians (41) 53.95%. Control group consisted of medical workers from Faculty Hospital in Martin and workers from Biotika factory in Martin. They were not exposed to any known genotoxic agents. Characteristic of exposed group and control is presented in Tab.1. 100 mitoses per person were microscopically analysed and frequency of total CAs and its subgroups: CTA-type and CSA-type of

Tab. 1. Characteristics of exposed group and control

	Exposed group	Control
Number (N)	76	76
Age (years±S.D.)	36.89±8.75	35.99 ±7.73
Exposure (years±S.D.)	11.75±9.35	
Sex (N) M/F	15 / 61	16 / 60
Smoking (N) S/NS	23 / 53	15 / 61
Job (N) physician/nurse	41 / 35	20 / 56

aberrations were evaluated. Cytogenetic analysis was performed according to AHEM [15]. Polymorphisms of DNA repair genes were performed by PCR-RFLP analysis. Genotypes were determined in direct process sequences of amplificied fragments. Gene *XPD* exon 23 (A C), kodon 751 (Lys751Gln): primers - F (forward): 5'-CCC CTC TCC CTT TCC TCT GTT-3'; R (reverse): 5'-GCT GCC TTC TCC TGC GAT TA-3'; restriction enzyme *Pst*I; size of fragments: 1) normal homozygote (Lys751Lys) - 290+146 bp; 2) heterozygote (Lys751Gln) - 290+227+146+63 bp; 3) variant homozygote (Gln751Gln) - 227+146+63 bp. Gene *XPG* exon 15 (G C), kodon 1104 (Asp1104His): primers - F (forward): F: 5'-TGG ATT TTT GGG GGA GAC CT-3'; R (reverse): 5'-CGG GAG CTT CCT TCA CTG AGT-3'; restriction enzyme *Hsp*92II; size of fragments: 1) normal homozygote (Asp1104Asp) - 159 bp; 2) heterozygote (Asp1104His) - 59+100+159 bp; 3) variant homozygote (His1104His) - 59+100 bp. Gene *XPC* exon 15 (A C), kodon 939 (Lys939Gln): primers - F (forward): 5'-GAT GCA GGA GGT GGA CTC TCT-3'; R (reverse): 5'-GTAGTGGGGCAGCAGCAACT-3'; restriction enzyme *Pvu*II; size of fragments: 1) normal homozygote (Lys939Lys) - 281 bp; 2) heterozygote (Lys939Gln) - 150+131+281 bp; 3) variant homozygote (Gln939Gln) - 150+131 bp. The peripheral blood sampling was realized within the specialised medical examinations. All principles of personnel data protection were accepted in presented study. Statistical analysis was performed by program Statgraphics, version 7 (Manugistics, Cambridge, MA). Nonparametrical Mann-Whitney U-test was used for testing of differences between the groups and analysis of variance (ANOVA) for testing of relationships between chromosomal aberrations and genotypes.

RESULTS

We detected statistically higher frequency of total CAs in exposed group (Tab. 2) in comparison to control (2.53±1.46% vs. 1.26±0.93%, Man-Whitney U-test, P=0.0008). In exposed group we found significant difference between CTA-type and CSA-type of aberrations (0.61±0.83% vs. 1.92±1.38%, Man-Whitney U-test, P=0.0009). Evaluating the role of polymorphisms of *XPD* gene and *XPC* gene, we observed the increase of total CAs, CTA-type and CSA-type in presence of variant allele. These differences were not statistically significant (Fig. 1 and Fig. 2). In *XPG* gene was not detected any difference nor in total CAs, neither in their types when variant allele C was present (Fig. 3).

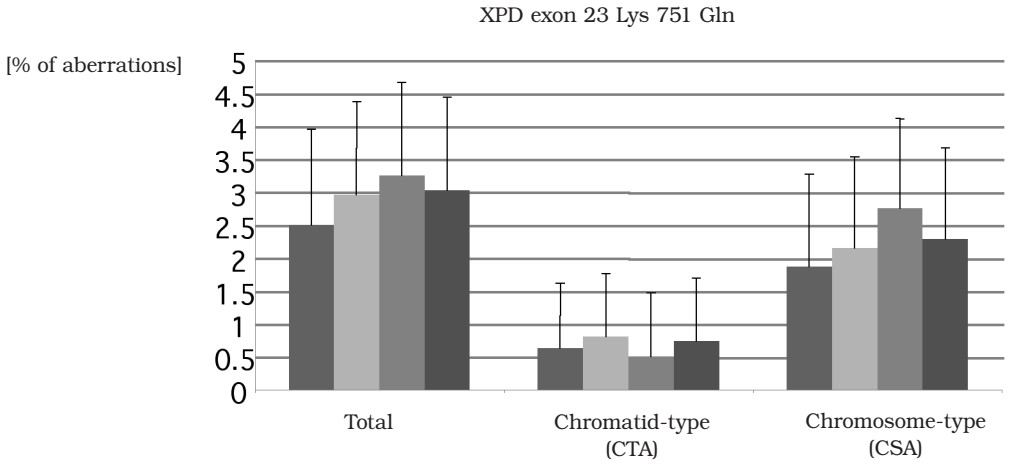
Tab. 2. Number of total chromosomal aberrations, chromatid-type (CTA) and chromosome-type (CSA) in exposed group and control

	Total CA'	Chromatid-type (CTA) %±S.D.	Chromosome-type (CSA) %±S.D.
Exposed group	2.53±1.46***	0.61±0.83	1.92±1.38***
Control	1.26±0.93	0.53±0.62	0.73±0.81

*** P = 0.0008 Total CA: exposed vs.control; *** P = 0.0009 Exposed group: CTA vs.CSA

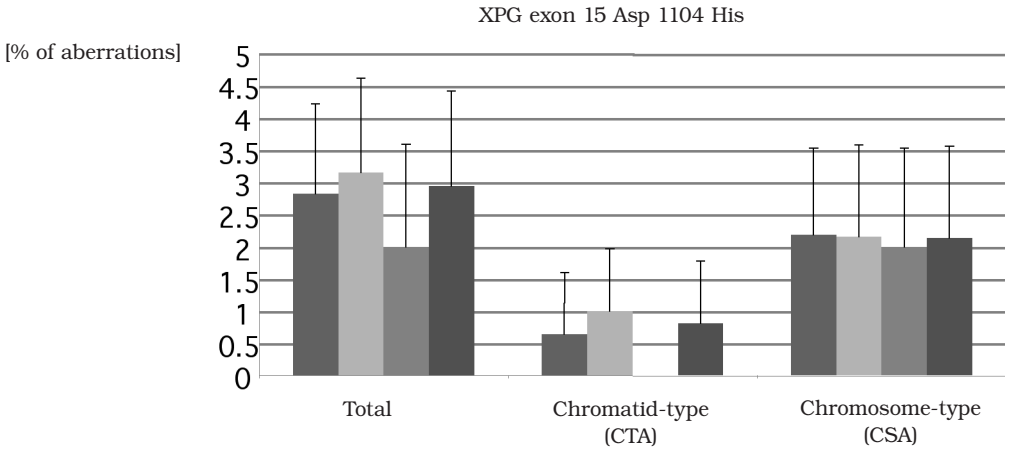
DISCUSSION

Anaesthesiology workers are exposed to volatile anaesthetics during their occupational activities. In many papers the increase of aberrant cells in operating rooms workers is presented [10, 11, 16]. The increased number of chromosomal aberrations in exposed persons can be caused by unsuitable conditions in operating rooms (e.i. not effective or inadequately



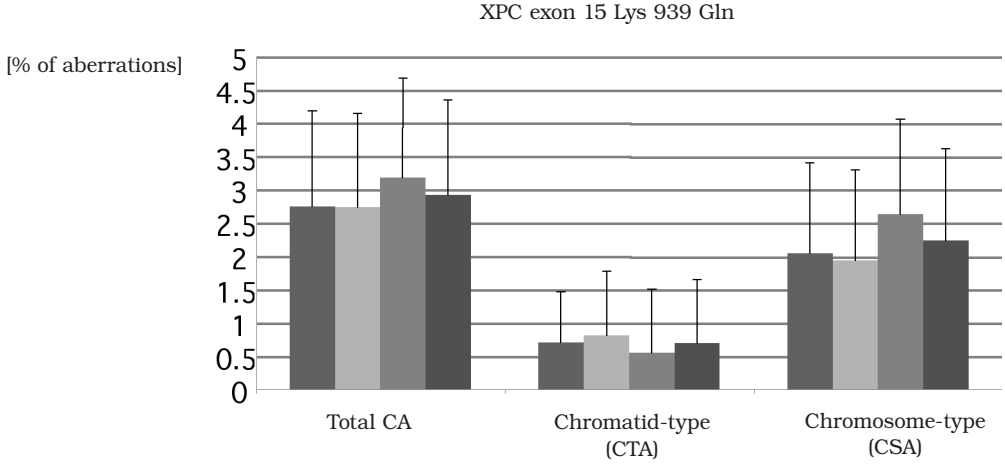
	Total	Chromatid-type (CTA)	Chromosome-type (CSA)
WT (n=16)	2,5	0,63	7,87
HET (n=26)	2,96	0,81	2,15
VAR (n=8)	3,25	0,5	2,75
HET+VAR (n=34)	3,3	0,74	2,29

Fig. 1. Number of total CA, CTA-type and CSA-type in gene *XPD*



	Total	Chromatid-type (CTA)	Chromosome-type (CSA)
WT (n=33)	2,82	0,64	2,18
HET (n=13)	3,15	1	2,15
VAR (n=3)	2	0	2
HET+VAR (n=16)	2,94	0,81	2,13

Fig. 2. Number of total CA, CTA-type and CSA-type in gene *XPG*



	Total CA	Chromatid-type (CTA)	Chromosome-type (CSA)
WT (n=23)	2,74	0,7	2,04
HET (n=15)	2,73	0,8	1,93
VAR (n=11)	3,18	0,55	2,63
HET+VAR (n=26)	2,92	0,69	2,23

Fig. 3. Number of total CA, CTA-type and CSA-type in gene *XPC*

effective air circulation, operating rooms without air-conditioning) in comparison to control. Many epidemiologic studies pointed out situation, that concentration of anaesthetics without effective air circulation is markedly higher than occupational exposure limits [17]. The extensive research of employment conditions showed, that in operating rooms with 10-12 multiple air circulation only, the concentration of halothane can be decreased to 50-80 $mg.m^{-3}$ [18]. The role of gene-environmental interactions has been frequently discussed for last years and the research results have pointed on their relation to diseases formation in some individuals. It is believed that this interaction can be influenced by gene polymorphism. For the determination of CAs frequency in human lymphocytes we analysed specific polymorphisms of DNA repair genes. We evaluated polymorphism of genes for nucleotide excision repair: *XPD*, *XPG* and *XPC*. In genes *XPD* and *XPC* we detected an increase of total chromosomal aberrations and its specific types in presence of variant allele, even in gene *XPG* we did not detected any difference in presence of variant allele C. The polymorphism of many genes included in BER or NER, and repair of double strand breaks is tightly connected with increased risk of tumours and DNA damages [19, 20, 21, 22, 23]. Genotoxic impact of gamma radiation reduces the ability of DNA repair process [24]. Vodicka et al. (2004) published the relationship between polymorphisms of DNA repair genes *XRCC1*, *XRCC3, XPD, XPG* and *XPC* and frequency of CAs and SCE. He detected the higher frequency of CAs in individuals with allele A in *XPD* gene exon 23, e.i. in individuals with genotype AA and AC [25]. It would be optimal to evaluate the individual susceptibility, and to determine „positive“ and „negative“ genotypes in order to minimize the risk of exposure in sensitive individuals. Unfortunately, it is not possible because we don't have sufficient evidence about responsible genes interactions consequences.

Presented results point out importance of individual susceptibility in assessment of genotoxic effects, in cases, when concentration of genotoxic agents usually doesn't exceed the occupational exposure limit.

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