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ASSESSMENT OF THE MECONIUM REMOVAL IN SURFACTANT VS. SALINE-LAVAGED RABBITS WITH MECONIUM ASPIRATION

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Abstract

Background and aim: Objective quantification of the amount of removed meconium is an important indicator of efficacy of approaches potentially improving meconium clearance from the lungs. The goal of this study was to assess the amount of removed meconium by surfactant vs. saline lavage in rabbits with meconium aspiration by spectrophotometry and meconium-crit methods.

Methods: Adult rabbits were anesthetized, tracheotomized, paralyzed and conventionally ventilated. Human meconium (25 mg/ml, 4 ml/kg b.w.) was instilled proportionally into the right and left lungs. When respiratory failure developed, surfactant (Surf, n=7) or saline (Sal, n=7) lavage at a dose of 10 ml/kg b.w. in 3 portions was performed. Lavage fluid was suctioned 1 and 5 min after the administration and meconium content in the lavage fluid was evaluated by spectrophotometry and meconium-crit methods.

Results: Animals of both groups were given comparable amount of meconium. Surfactant lung lavage removed significantly more meconium when evaluated by meconium-crit method ($P<0.05$), however, the difference between groups was not significant when evaluated by spectrophotometry ($P>0.05$).

Conclusions: The discrepancy between the results estimated by spectrophotometry and meconium-crit methods resulted from the different qualities evaluated by these two methods. Moreover, since water-soluble parts of meconium were effectively removed by both saline and surfactant lavage, surfactant improved the clearance of cholesterol-soluble fraction of meconium, too.

Key words: meconium, lung lavage, rabbit

INTRODUCTION

Despite combined therapy, meconium aspiration syndrome (MAS) is one of the most severe diseases in the term newborns. Aspiration of meconium causes obstruction of the airways, inactivation of pulmonary surfactant, inflammation and pulmonary vasoconstriction, that often leads to the respiratory failure.

Chemically, meconium can be extracted to the water-soluble (gastrointestinal enzymes, bile acids, bilirubin etc.) and chloroform-soluble (free fatty acids, cholesterol, neutral lipids etc.) fractions (1). Due to the high concentration of mucopolysaccharides, meconium is extremely adhesive to the airway walls, and clearing procedures such as airway suctioning and physiotherapy may be ineffective.

Lung lavage with diluted exogenous surfactant increased the meconium clearance from the lungs in several experimental studies (2-6). Improvement in oxygenation and survival after the surfactant lavage was found also in the newborns with MAS (7-9).

Although standardization of the lavage procedure in the terms of the number of lavages, volume of the administered fluid, and concentration and type of surfactant until now have not inferred to the final conclusion, amount of the removed meconium became an important indicator of efficacy of approaches potentially improving meconium clearance from the lungs.

Whereas the previous use of the methods objectively quantifying the meconium content in the recovered lavage fluid led to the controversial results, the goal of this study was to assess the amount of removed meconium by surfactant vs. saline lavage in rabbits with meconium aspiration by spectrophotometry and meconium-crit methods.

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METHODS

Meconium

Human meconium was collected from 20 healthy newborns, lyophilized, pooled and stored in –20 °C. Before use, meconium was suspended in 0.9 % NaCl at a concentration of 25 mg/ml.

Surfactant

Modified porcine surfactant (Curosurf, Chiesi Pharmaceutici, Italy) was diluted in saline to a phospholipid concentration of 10 mg/ml.

Design of experiments

Experiments were carried out in concordance with the basic ethical norms and NIH Publication No. 86-23, revised 1985. Rabbits of the mean body weight (b.w.) of 2.0 kg were anesthetized with intramuscular ketamine (Narkamon, Spofa, Czech Republic) at a dose of 20 mg/kg b.w. and xylazine (Rometar, Spofa, Czech Republic) at a dose of 5 mg/kg b.w., followed by intravenous ketamine at a dose of 20 mg/kg b.w./hour. Animals were then tracheotomized, paralyzed with pipecuronium bromide (Arduan, Gedeon Richter, Hungary) at a dose of 0.3 mg/kg b.w./30 min i.v. and ventilated conventionally by pressure-controlled ventilator Beat-2 (Chirana, Slovakia) with frequency of 30/min. Meconium at a dose of 4 ml/kg b.w. was instilled proportionally into the right and left lungs. Additional dose of meconium (25 mg/ml, 1 ml/kg b.w.) was given to animals not showing evidence of respiratory failure, defined as >30 % decrease in lung-thorax compliance and PaO_2<10 kPa at FiO_2 1.0, 15 minutes after the first dose. Animals were then lavaged with surfactant (Surf, n=7) or saline (Sal, n=7). The lavage fluid at a dose of 10 ml/kg b.w. divided into 3 equal portions was instilled into the tracheal cannula by syringe during positioning of the animal and suctioned using negative pressure of 60 kPa 1 and 5 min after the fluid administration. Volume of suctioned fluid was measured and meconium content in the lavage fluid was evaluated at the end of experiments by both spectrophotometry and meconium-crit methods.

Estimation of meconium removal by spectrophotometry

Basic suspension of 2.5 g of lyophilized meconium in 100 ml of saline was diluted to five different concentrations (2.5, 1.25, 0.625, 0.5, and 0.25 mg/ml). Samples were centrifuged at 40000xg for 1 hour and optical density was measured. Calibration curve expressing the linear relationship between the concentration of meconium and optical density of supernatant was constructed (Fig.1). In experiments, samples of the lavage fluid were centrifuged at 40000xg for 1 hour. The optical density (OD) values of supernatant read at 260 and 300 nm were added to the formula: $OD_{300} - (0.13)\ (OD_{260})$ (10). Meconium content in the lavage fluid was evaluated accord-

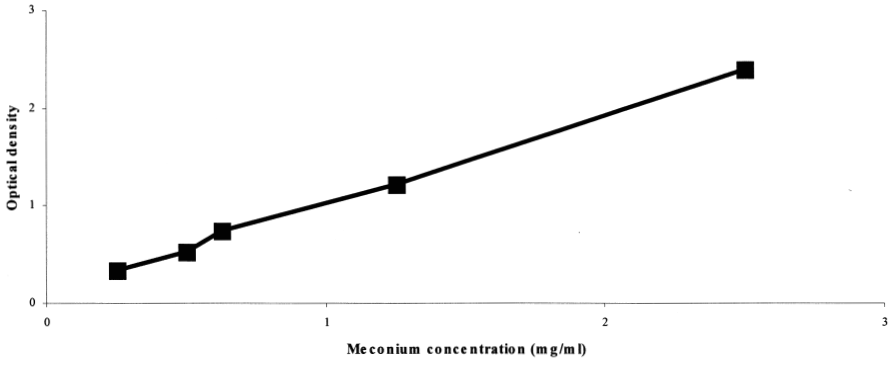

Fig. 1. Calibration curve for spectrophotometry.

ing to the calibration curve and expressed as a percentage of the total amount administered to an individual animal.

Estimation of meconium removal by meconium-crit method

Basic suspension of 3.0 g of meconium in 100 ml of normal saline was diluted to seven different concentrations (30, 20, 15, 10, 6, 3, and 2 mg/ml). Ten samples of each suspension were taken into the standard microhematocrit glass tubes and centrifuged at 10 000 rpm for 5 minutes (11). The meconium-crit was then measured directly as the hematocrit, i.e. as a ratio of the solid content to the total volume, and mean values were calculated. Calibration curve showing the relationship between the concentration of meconium and meconium-crit value was constructed (Fig. 2). In experiments, three samples of the lavage fluid were taken into the microhematocrit glass tubes and centrifuged at 10 000 rpm for 5 min. Percentage of solid content was read. Average value of removed meconium was expressed as a percentage of the total amount administered to an individual animal.

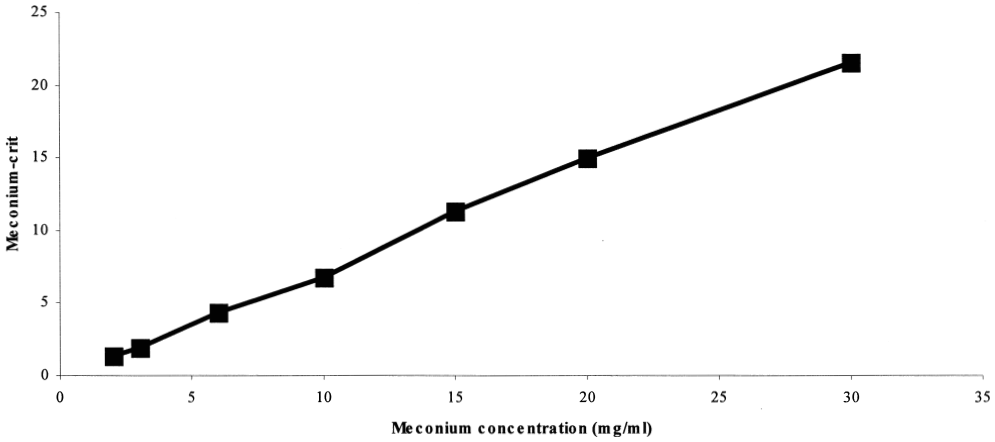

Fig. 2. Calibration curve for meconium-crit method.

STATISTICS

Between-group differences were analysed using Mann-Whitney's test. A *P*-value<0.05 was considered statistically significant. Data are expressed as means±SD.

RESULTS

No significant differences in the body weight or initial values of measured parameters were found between the groups (*P*>0.05). Animals of both groups were given comparable amount of meconium – 100 mg/kg b.w. (25 mg/ml, 4 ml/kg), i.e. total mean value 110.7±13.4 mg of meconium per kg b.w.

As shown by spectrophotometry, amount of meconium removed by saline lavage was 12.7±2.0 mg/kg b.w., i.e. 11.0±1.1 % of administered meconium, while by surfactant lung lavage it was 15.1±3.1 mg/kg b.w., i.e. 13.6±2.5 % of administered meconium. By meconium-crit method, saline lavage removed 7.9±2.8 mg/kg b.w. of meconium, i.e. 5.4±2.2 % of instilled meconium, and surfactant lavage removed 19.1±9.7 mg/kg b.w., i.e. 11.1±4.5 % of administered meconium. Statistical between-group comparison revealed that amount of meconium removed by surfactant lung lavage compared to saline lavage was higher, when evaluated by meconium-crit method than by spectrophotometry (Fig. 3, Fig. 4).

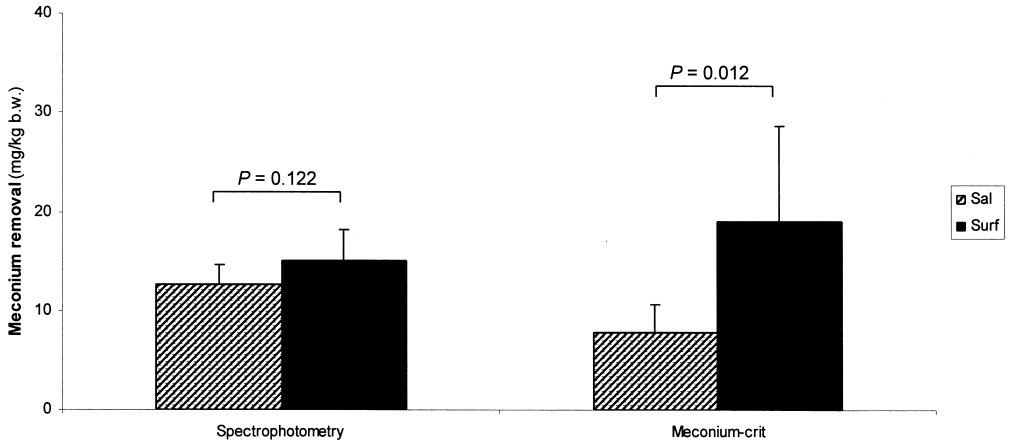

Fig. 3. Meconium removal (mg/kg b.w.) evaluated by spectrophotometry and meconium-crit methods in saline- (Sal) and surfactant-lavaged (Surf) groups.

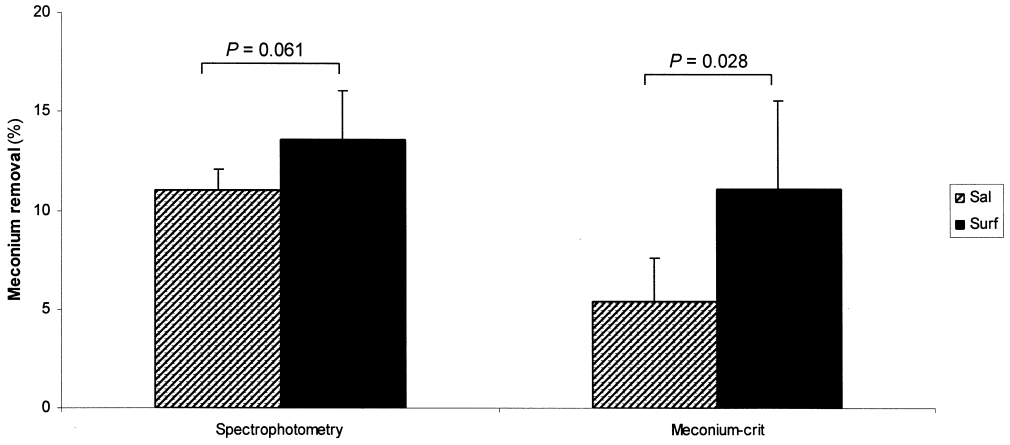

Fig. 4. Meconium removal (%) evaluated by spectrophotometry and meconium-crit methods in saline- (Sal) and surfactant-lavaged (Surf) groups.

DISCUSSION

In the previous experimental studies, meconium clearance from the lungs by surfactant lung lavage was evaluated either by meconium-crit method or by spectrophotometry. However, while Ohama and Ogawa (2) and Lam et al. (3) using meconium-crit method found significantly more meconium removed by surfactant lavage than saline lavage, Cochrane et al. (10) using spectrophotometry found comparable amount of meconium removed by saline and surfactant lavage. Discrepancy between the results obtained by these two methods yielded with effort to evaluate meconium removal by surfactant vs. saline lung lavage in a rabbit model of meconium aspiration syndrome using both methods.

In our study, spectrophotometry and meconium-crit methods showed higher meconium clearance by diluted exogenous surfactant than by saline lavage. However, the between-group differences were more pronounced, when evaluated by meconium-crit method. Our results are thus comparable with the results of the authors mentioned above. Meconium removal by surfactant lavage evaluated by meconium-crit method was significantly higher compared to saline lavage,

in concordance with Ohama and Ogawa (2) and Lam et al. (3). On the other side, in meconium removal evaluated by spectrophotometry there was no significant difference between the groups, comparably to Cochrane et al. (10). We suppose that this finding results from the different qualities estimated by spectrophotometry and meconium-crit methods. While meconium-crit method is based on the measurement of total solid content in the lavage fluid, spectrophotometric method measures the optical density of greenish-coloured meconium in the fluid.

Rather high meconium removal evaluated by spectrophotometry, but relatively low by meconium-crit method indicates that saline lavage removes especially pigmented parts of meconium from the lungs, which are soluble in water such as gastrointestinal enzymes, bile acids and bilirubin. On the other side, exogenous surfactant acts as a detergent lowering the surface tension of highly tenacious meconium. Therefore, we found higher removal of meconium pigments, but particularly high clearance of those parts of meconium, which are hardly cleared by saline – mucopolysaccharides, free fatty acids, triglycerides, cholesterol etc., estimated by meconium-crit method.

Dargaville et al. (4) recently evaluated clearance of both meconium pigments and meconium solids in the piglet model of MAS, however, with different methods. Dry weight of the purified meconium pellet was determined after repeated centrifugation of the lavage fluid and dessication, and meconium pigment concentration in the supernatant was analysed by fluorescent spectrophotometry. Similarly to our results, the percentual removal of pigments by both saline and surfactant lavage was higher than the removal of meconium solids despite another type and concentration of the used surfactant and larger aliquot volume improving distribution of the lavage fluid throughout the lungs.

According to the presented results, we can conclude that discrepancy between the results obtained by spectrophotometry and meconium-crit methods is caused by the different qualities measured by these two methods. While saline lavage removes mostly water-soluble pigmented parts of meconium, exogenous surfactant improves the clearance of both water-soluble and cholesterol-soluble fractions of meconium lowering the surface tension of tenacious substances present in meconium.

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VISUALISATION OF CARDIOVASCULAR DYSREGULATION IN YOUNG PATIENTS WITH TYPE 1 DIABETES MELLITUS BY POINCARÉ PLOT

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Abstract

The noninvasive assessment of spontaneous physiological parameters variations can provide valuable information about control systems involved in their complex regulation. Time series analysis is usually performed in time and frequency domains – the so called linear methods. Inspired by effort to apply nonlinear time series analysis into cardiovascular variability signals, the aim of this study was to compare heart rate and blood pressure variabilities (HRV and BPV) between young patients with type 1 diabetes mellitus (DM) and control subjects using Poincaré plot.

Patients with type 1 DM (10 females, 7 males) aged 12.9 – 31.5 years (mean ± SEM: 22.4 ± 1.0 years) were investigated. The control group consisted of 17 healthy probands matched for sex and age. The HRV and BPV were analysed in time domain (mean, standard deviation - SD) and using quantitative analysis of Poincaré plot pattern measures during supine rest.

In young patients with type 1 DM, significant reduction of all measured Poincaré plot parameters constructed from R-R intervals was found. However, no significant difference between groups in BPV Poincaré plot measures was observed.

In conclusion, HRV Poincaré plot was able to reveal beat-to-beat HRV abnormalities. Poincaré plot can provide information potentially usable for diagnosis and prognosis in visually understandable manner. We suggest that parasympathetic dysfunction in cardiac regulation occurs earlier than dysregulation of sympathetic control of the vessels in young diabetics.

Key words: heart rate variability, blood pressure variability, diabetes mellitus, Poincaré plot

INTRODUCTION

The noninvasive assessment of spontaneous physiological parameters variations in time can provide valuable information about control systems involved in their complex regulation. Repeatedly measured values of any assessed parameter form *time series* that can be regarded as a signal encompassing information about structure and status of dynamical system that generates and modifies given parameter. *Time series analysis* is able to acquire data about normal dynamical system as well as system changes during pathological circumstances with clinically important applications (diagnosis, prognosis) (1, 2).

Time series analysis of physiological parameters is usually performed in time and frequency domains – the so called linear methods. Time domain parameters based on basic statistical parameters (e.g. mean value, standard deviation) provide summarized information on short- and long-term variability in time series, but did not comprise information concerning oscillations` patterns (3). These parameters are sensitive to artefacts and require gaussian distribution of measured parameter. Time series analysis in frequency domain (spectral analysis) enables to quantify cyclic oscillations (e.g. respiratory sinus arrhythmia). The nonperiodic oscillations are ignored and usually regarded as a noise (1,4).

Cardiovascular control system components (baroreceptors, chemoreceptors, sympathetic and parasympathetic nervous system, cardiac pacemaker, smooth muscles of blood vessels, etc.) interact in a complex manner. These interactions are usually not linear – output is not proportional to input (e.g. heart rate changes are not directly proportional to blood pressure changes). The nonlinear systems are able to generate very complex signals that cannot be distinguished from noise using linear time series analysis tools. Therefore, there is an ongoing

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effort to apply nonlinear time series analysis into cardiovascular variability signals (1, 3, 4, 5).

Application of new mathematical tools based on nonlinear dynamics to heart rate and blood pressure variabilities analysis provides supplementary information about systems involved in cardiovascular parameters changes. Computing of the commonly used nonlinear parameters (Lyapunov exponent, correlation dimension) from the signal requires relatively long and stationary signals which are difficult to obtain from living animals and humans. The graphical analysis by Poincaré plot is increasingly used because it can be performed from shorter quasi-stationary records. Poincaré plot is on the boundary between linear methods of biosignal analysis and tools based on nonlinear dynamics – the principle of its construction is taken from the nonlinear dynamics theory, but parameters used for its quantification are essentially linear (6). In addition, Poincaré plot enables to display information about beat-to-beat variability of heart rate in compact visual format which is easy to read (4,7).

Autonomic neuropathy and cardiovascular dysregulation are usually regarded as the late complications of diabetes mellitus (DM). However, autonomic nervous system dysregulation can be detected by modern sensitive methods even in early phases of DM (8, 9, 10, 11). Early diagnosis of autonomic neuropathy is important because the mortality of the patients with this complication is markedly higher (12, 13).

The reduction of spontaneous heart rate variability (HRV) is regarded as one of the early signs of cardiac autonomic neuropathy (8, 14). HRV originates predominantly from parasympathetic nervous traffic oscillations (15) and therefore HRV analysis can provide information about vagal component of the autonomic nervous system (16). On the other side, smooth muscles of the vessels and hence peripheral vascular resistance are under dominant sympathetic nervous system control. Therefore, the analysis of blood pressure variability (BPV) can be useful for detection of sympathetic dysfunction (16, 17, 18).

Relatively few studies were focused on cardiovascular dysregulation in adolescents and young adults with type 1 DM, although a multicentric study EURODIAB has found the cardiac autonomic neuropathy in 19% of diabetics in the age group 15-29 years (19). Short-term BPV was analysed in diabetic patients only in time domain (20, 21, 22) and, to our knowledge, Poincaré plot of BPV in young diabetics has not been used yet.

The aim of the study was to compare heart rate and blood pressure variabilities between young patients with type 1 DM and control subjects using Poincaré plot.

METHODS

Subjects

We have investigated 17 young patients with type 1 DM (10 females, 7 males) aged 12.9 – 31.5 years (mean ± SEM: 22.4 ± 1.0 years). The mean duration of DM was 12.4 ± 1.2 years. The control group consisted of 17 healthy probands matched for sex and age. All subjects were familiarised with investigation protocol and they gave informed consent. Probands were instructed to avoid smoking and drinking alcoholic beverages for 24 hours before investigation. Several basic study groups characteristics are presented in Table 1.

Table 1: Study groups characteristics (control group – C, group of patients with type 1 diabetes mellitus - DM). Values are presented as mean ± SEM and P-values were obtained using Mann-Whitney U-test. Asterisk indicate significant (P<0.05) between-groups difference.

	C	*DM*	*P*
Age (years)	21.9 ± 0.9	22.4 ± 1.0	0.617
Body Mass Index (kg m^{-2})	21.2 ± 0.7	23.1 ± 0.7	0.033*
Plasma glucose (mmol l^{-1})	4.8 ± 0.1	10.2 ± 1.4	0.001*
HbA1c (%)	4.8 ± 0.1	9.6 ± 0.4	0.001*

Protocol

The length of R-R intervals was measured using telemetric system (VariaCardio TF4, Sima Media, Olomouc, Czech Republic) where ECG signal (sampling freqency 1000 Hz) from thoracic belt with integrated electrodes was transferred into PC for further analysis. Systolic blood pressure (SBP) was monitored beat-to-beat using volume-clamp method (23) by Finapres 2300 (Ohmeda, USA). The finger cuff of appropriate size was wrapped around middle phalanx of the third finger of the left hand. The finger was passively maintained at the heart level to avoid blood pressure distortion caused by hydrostatic pressure changes. Analog output of the Finapres was transferred into PC by analog-digital convertor PCL-711 (Advantech Co., Taiwan) with the sampling frequency of 500 Hz. The SBP values were obtained on-line using specially developed software and stored in PC for subsequent analysis.

All subjects were investigated in quiet room from 7.30 to 12.00 a.m. The thoracic belt with ECG electrodes and finger cuff of Finapres device were applied after 10 minutes in sitting position. Then, the subject was in supine position on the bed during next 70 minutes of the continuous recording of the cardiovascular parameters. We have asked the probands to avoid voluntary movements and speaking as much as possible.

Data analysis

HRV and BPV analysis was performed off-line in selected interval (interval started 30 min after reclining, the length of interval was 600 s) of the records using special software.

In analysed time interval we quantified several basic time domain HRV and BPV parameters: mean R-R interval duration (mean RR), standard deviation of the R-R intervals (SDRR), mean SBP and standard deviation of SBP values (SDSBP).

Poincaré plot is the semiquantitative tool for physiological parameters variability analysis (most commonly used in for HRV analysis) which enable to assess their non-random beat-to-beat changes. The HRV Poincaré plot is the scatterplot of current R-R interval length against the R-R interval length immediately preceding it and provides visually understandable information about both overall and beat-to-beat HRV. If the heart rate rhythm is regular then the points in Poincaré plot are located closely around the line of identity (axis of the 1st quadrant) (4, 24). Analogously, BPV Poincaré plot is the scatterplot where x-coordinate of each point is the current SBP and y-coordinate is the SBP value during previous heart beat.

Poincaré plots of HRV and BPV were constructed from resampled R-R and SBP time series (at 1 Hz) and quantitatively analysed. Quantitative analysis of the Poincaré plot patterns (Fig. 1) was performed using self-developed software. The widths of Poincaré plot pattern at 10th, 25th, 50th, 75th and 90th percentiles of R-R intervals (SBP) distribution – W10, W25, W50, W75 and W90 – were quantified according to Schechtman *et al.* (1992) and Silke *et al.*(1999). This approach enables to quantify beat-to-beat heart rate (and SBP) changes at given "basal" heart rate (SBP)

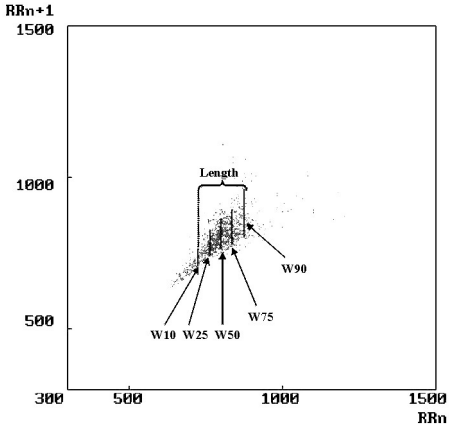

Fig. 1: Poincaré plot analysis with quantified measures

level - common comet-like shape of Poincaré plot pattern is caused by higher beat-to-beat variability around longer R-R intervals (higher widths at 75th and 90th percentiles). The length of the Poincaré plot was defined as the difference between 10th and 90th percentiles of R-R intervals (SBP) distribution.

Statistics

Nonparametric tests were used due to non-gaussian distribution of the HRV nad BPV parameters. The non-gaussian distribution of the variables was ascertained using the Lilliefors test. Between-groups comparisons (DM vs control group) were performed using Mann-Whitney U-test. All inferential statistics were considered significant at $P<0.05$ level and values are presented as mean ± SEM.

RESULTS

Figure 2A illustrates differences in heart rate recordings between representative control subject (left) and patient with DM (right). Marked reduction in magnitude of overall variability and diminished beat-to-beat fluctuations were found in diabetic subject. Figure 2B shows similarity of spontaneous SBP oscillations in healthy control subject (left) and diabetic patient (right).

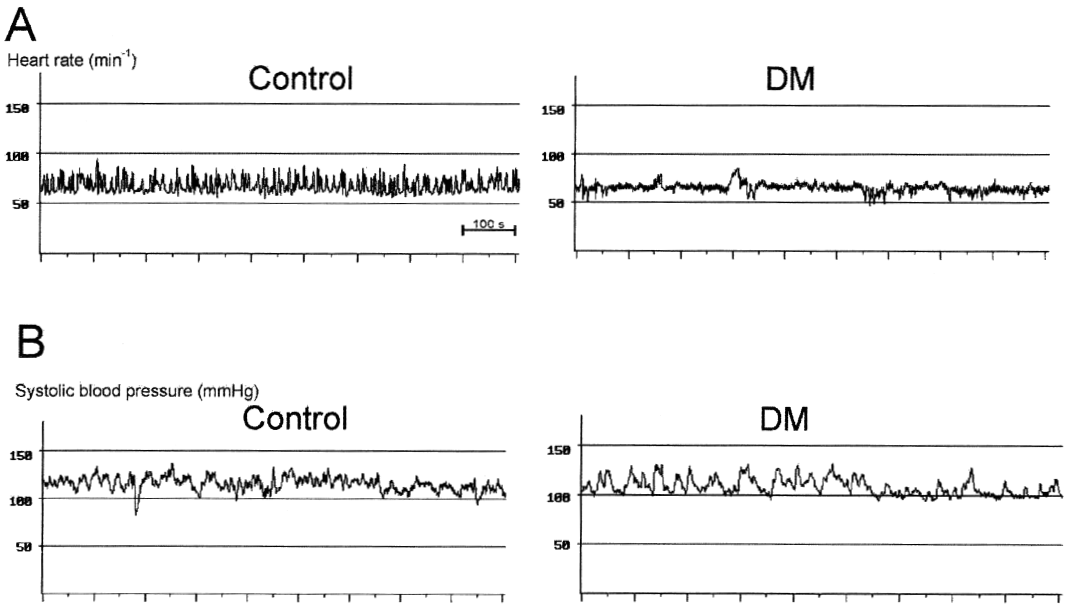

Fig.2: Representative heart rate (A) and systolic blood pressure (B) records from control subject (left) and patient with diabetes mellitus (right). Marked reduction of beat-to-beat heart rate oscillations amplitude in diabetic subject was observed. Blood pressure variability was similar in diabetic subject compared to control subject.

Heart rate variability

Although mean RR tended to be lower in DM group, no statistically significant difference in mean R-R interval length between groups was found (control group: 903 ± 30 ms; DM group: 825 ± 29 ms ; P=0.058). Significantly reduced overall HRV was reflected in SDRR (control group: 94 ± 10 ms; DM group: 56 ± 8 ms ; P=0.017).

The example of typical HRV Poincaré plots for representative control subject and young diabetic patient are shown in Figure 3. Marked reduction in all measures of Poincaré plot pattern can be clearly seen. Statistical analysis showed, that the length and all quantified widths (W10 to W90) of the Poincaré plot pattern constructed from the resampled R-R intervals were significantly lower in DM group compared to control group (Figure 4).

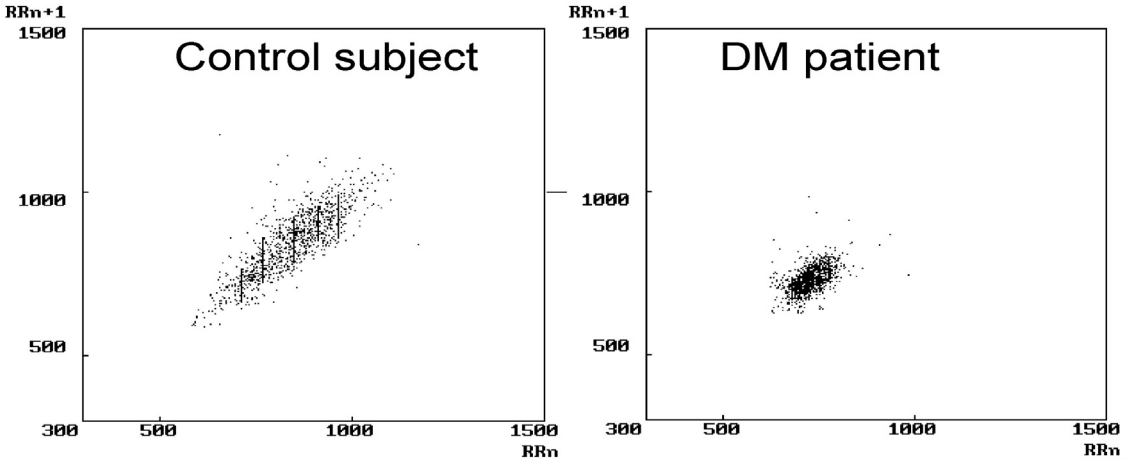

Fig. 3: HRV Poincaré plots illustrate differences in beat-to-beat heart rate variability between a healthy control subject and a patient with DM. Marked reduction in all Poincaré plot indices was found in young DM patients.

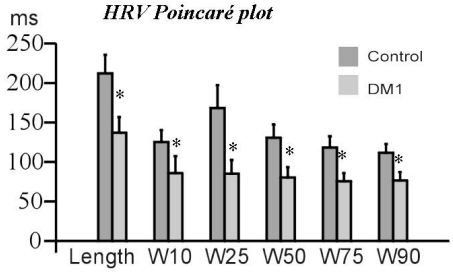

Fig. 4: Heart rate variability analysed using Poincaré plot in control subjects (dark gray) and patients with DM (light gray). Bars and error lines represent mean and SEM, respectively. Asterisks indicate significant ($p<0.05$) between group differences.

Blood pressure variability

No statistically significant difference in mean SBP (control group: 117 ± 3 mmHg; DM group: 116 ± 3 mmHg ; P=0.597) and SDSBP (control group: 6.9 ± 0.6 mmHg ; DM group: 7.0 ± 0.4 mmHg; P=0.955) between groups was found.

Similarity of BPV Poincaré plot patterns in representative control subject and diabetic patient are illustrated in Figure 5. No significant differences between groups in BPV Poincaré plot parameters were found (Figure 6).

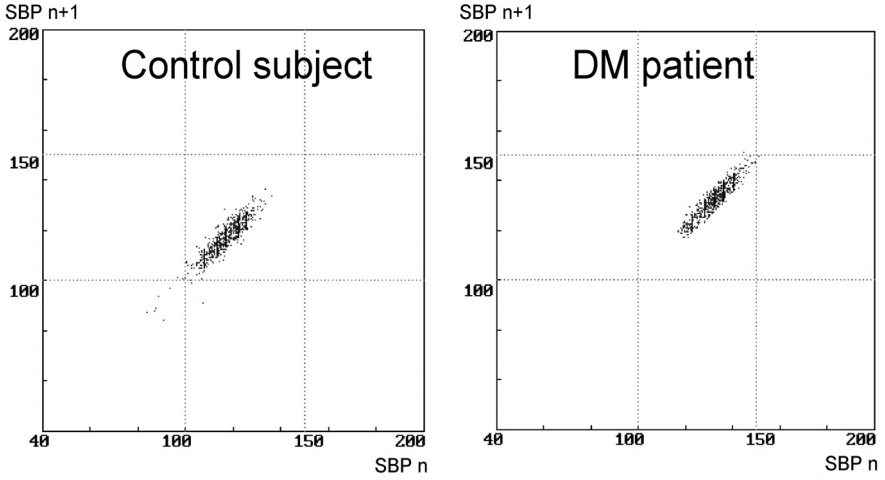

Fig. 5: BPV Poincaré plots show similarity of systolic blood pressure changes in both analysed groups.

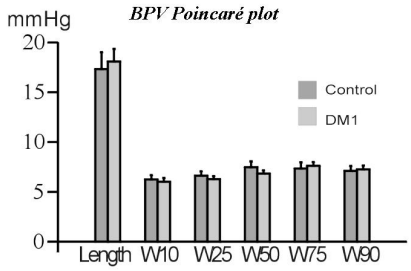

Fig. 6: BPV Poincaré plot parameters in control subjects (dark gray) and patients with DM (light gray). Bars and error lines represent mean and SEM, respectively.

DISCUSSION

HRV Poincaré plot changes during various physiological manoeuvres and diseases

Kamen et al. (25) assessed changes of Poincaré plot pattern constructed from R-R intervals during head-up tilt test and pharmacological interventions. Head-up tilt test and parasympathetic blockade by atropine were accompanied by reduced Poincaré plot length and width. On the other hand, transdermal application of scopolamine (parasympathetic activator) increased Poincaré plot measures. Parasympathetic nervous system influence on Poincaré plot parameters was analysed also by Tulppo et al. (26). Increasing doses of atropine caused predominantly reduction of pattern width although the decrease in length was also significant. These findings indicate that Poincaré plot pattern width reflecting beat-to-beat HRV can be regarded as an cardiac parasympathetic system activity index.

Poincaré plot was able to detect HRV abnormalities also during pathological circumstances. In children who later succumbed to sudden infant death syndrome (SIDS), significant reduction in Poincaré plot width on 90th percentile of R-R interval distribution was found (27). Reduced width and length of Poincaré plot pattern was found in patients with chronic ischemic heart disease. Potentially useful for the patients` prognosis can be observation that in post- myocardial infarction patients with ventricular tachyarrhythmias is reduced length and width of pattern compared to patients without arrhythmias (28). Epidemiological study proved that Poincaré plot parameters provide independent prognostic value concerning sudden death in patients with chronic cardiac failure (29).

Heart rate variability in DM patients

The characteristic findings in adult diabetic patients with cardiovascular autonomic neuropathy are resting tachycardia and mostly reduced HRV, which is the earliest sign of cardiac autonomic dysfunction (8). In our study we did not find any significant difference in mean heart rate (represented by its reciprocal value – mean RR) in supine position between diabetic patients and control group, but we found reduced overall HRV (reduced SDRR) in young patients with DM. These findings are in agreement with other studies (13, 14, 20, 30, 31) where nonsignificant difference in resting mean heart rate was often accompanied by significant differences in conventional (time and frequency domains) HRV parameters in diabetic patients.

From the Poincaré plot parameters, young diabetic patients had all measures of the pattern reduced. This was not surprising, beacause the decreased overall HRV (represented by the length of Poincaré plot pattern) and decreased beat-to-beat HRV (represented by the width) was expected in DM patients. The studies of Kamen *et al.*(25), Tulppo *et al.* (26) and others confirmed the reduction of Poincaré plot pattern measures in diseases and manoeuvres linked with parasympathetic nervous system activity inhibition. As known before (25, 26, 32), we have also observed significant positive correlations between Poincaré plot measures and mean R-R interval (results not shown). Despite this fact, Poincaré plot can provide supplementary information about beat-to-beat variability at various heart rates which is unavailable by time and frequency domain analysis (4, 28, 33, 34). These findings indicate dysfunction of the parasympathetic component of autonomic nervous system in DM patients, because the short-term HRV is mostly mediated by vagal nerve discharge changes (15, 35).

Blood pressure variability in DM patients

Direct intraarterial measurement of blood pressure is the most precise method for its continuous monitoring. However, the usage of this method is markedly limited by its invasiveness (36). The volume-clamp method is the only alternative for noninvasive beat-to-beat blood pressure monitoring (23, 37). Although absolute values of the blood pressure obtained using this method can be distorted (overestimated systolic and underestimated diastolic blood pressures), volume-clamp method is able to reliably follow spontaneous blood pressure oscillations (38).

Short term blood pressure changes are mediated mostly by sympathetic nevous system and therefore the analysis of short term BPV has been taken as_more sensitive for detection of sympathetic dysregulation than HRV analysis (16, 17, 18).

Scaramuzza *et al.* (10) observed significant reduction in mean peripheral SBP values measured by Finapres in adolescents with type 1 DM. However, we did not find any significant difference in mean SBP between young DM patients and control group.

Several authors observed dysfunction of sympathetic control of the vessels in diabetic patients manifested as an reduction of spectral power in low frequency band in systolic blood pressure and skin blood flow signals (12, 39). Mésangeau *et al.* (40) found reduced standard deviation of blood pressure signal in animals with induced type 1 DM. In contrast, Chau *et al.* (20) did not observe changes in overall BPV in DM patients compared to control group. In our study no significant changes in overall systolic BPV were found.

We hypothesized that Poincaré plot analysis could be able to detect subtle abnormalities in beat-to-beat SBP control at various basal SBP levels in young patients with DM. However, no significant changes in BPV quantified by Poincaré plot pattern measures was observed in our group of patients compared to control group. We suggest that the significant sympathetic nervous system dysfunction was not present in our group of young diabetic patients.

In conclusion, Poincaré plot constructed from R-R intervals was able to reveal beat-to-beat HRV abnormalities. Poincaré plot can provide information potentially usable for diagnosis and prognosis in visually understandable manner. No significant difference between DM group and control group was observed in BPV quantified by Poincaré plot and standard deviation of SBP. We suggest that parasympathetic dysfunction in cardiac chronotropic regulation occurs earlier than dysregulation of sympathetic control of the vessels in young diabetics.

Acknowledgements

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STIMULATION OF DISTAL ESOPHAGUS HAS NO INFLUENCE ON THE COUGH REFLEX IN AWAKE GUINEA PIGS WITH EXPERIMENTAL ALLERGIC RHINITIS

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Abstract

Intranasal stimulation with capsaicin and experimentally induced rhinitis enhances cough response probably via central plasticity of the nucleus of the solitary tract neurons due to strong afferent inputs from the nose (1). Isolated intraesophageal administration of capsaicin did not induce any changes in the cough response (2).

This study was designed to test whether combinations of afferent inputs from the nose and esophagus could enhance the intensity of citric acid (CA) induced cough in guinea pigs.

16 male TRIK strain guinea pigs were sensitised by ovalbumin. 21 days after sensitization was confirmed by skin tests. Experimental allergic rhinitis was induced in these animals by intranasal ovalbumin challenge. After development of nasal symptoms the animals of experimental group (E) were challenges with intraesophageal (IE) capsaicin (1000 μM, 250 μl). Controls received IE saline. Cough was induced during IE stimulation both in E and C groups by citric acid and compared to baseline cough response tested in the beginning of the study. Number of coughs was analysed from pneumotachographic records of airflow changes typical for cough.

The number of CA induced coughs in C was increased after the induction of rhinitis and IE administration of saline [4 (1.5-5) vs 6 (3.5-8), $p < 0.05$]. Similar result was obtained for group of E animals with rhinitis IE challenged with capsaicin [3(2-4) vs 5 (5-6.5), $p < 0.05$]. This increase of the number of coughs is due to rhinitis. We did not find any differences in the cough response that could be ascribed to IE administration of saline or capsaicin ($p = 0.26$).

Conclusion: Intraesophageal administration of capsaicin did not affect CA induced cough in guinea pigs suffering from allergic rhinitis.

Key words: cough plasticity, allergic rhinitis, intraesophageal capsaicin challenge, guinea pig cough models

INTRODUCTION

In 1977, Irwin and coworkers (3) published a comprehensive review proposing that the cause of cough could be determined if a systematic evaluation was performed of the anatomic locations where cough receptors reside in the afferent limb of the cough reflex. The afferent limb includes the vagus nerve, which also innervates the esophagus. Using this protocol, Irwin and coworkers (3) reported a prospective trial in which the cause of cough was found in all subjects and specific therapy toward the cause resulted in cough resolution in 98% of subjects. Gastroesophageal reflux was the etiology of cough in 10% of their population when the diagnosis was made by history, endoscopy, or barium esophagogram (4).

The utility of the anatomic diagnostic protocol for chronic persistent cough and the importance of GERD as a cause of cough is proved (5, 6, 7).

There are two proposed mechanisms of GERD-associated cough: (A) acid in the distal esophagus stimulating an esophageal-tracheobronchial cough reflex, and (B) microaspiration or macroaspiration of esophageal contents into the larynx and tracheobronchial tree. A number of studies have been done to examine potential mechanisms affecting cough reflex in patients suffering from pathological gastroesophageal reflux (8, 9, 10).

In addition to the discussed points of pathogenesis of chronic cough in patients with GERD, there may be at least one important mechanism involved. It is supposed that activity of central cough pattern generator (CPG) is under plasticity of numerous stimuli, conducted to the brainstem via afferent nerve connections of these afferents with neuronal circuits (network) responsible for cough. Afferent stimuli originated in the nasal mucosa in patients suffering from rhinitis

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and those originated in esophagus in patients with GERD and subsequent convergence of these afferent inputs in the nucleus of the solitary tract (nTs) could be responsible for cough in this group of patients, although the pathological process is localized outside the respiratory tract area, from which the cough could be clearly elicited (11).

The simplest hypothesis explaining the cough enhancement by reflux is that the intense activation of sensory nerves in esophagus either directly triggers cough and/or sensitizes the cough reflex. These changes would result in the inappropriately active cough reflex leading to chronic coughing.

In our previous study we approached this hypothesis in the guinea pig cough models. Citric acid–induced cough was elicited during stimulation of the esophageal afferent nerves in the intact esophagus or esophagus with injured mucosa. We found that in either model the transient introduction of the nociceptive sensory nerve activator capsaicin into the esophagus did not trigger cough or affected cough induced by citric acid. Our results suggest that acute localized stimulation of esophageal mucosal nerves is not sufficient to trigger and/or enhance cough in guinea pigs.

There is also suggestion that stimulation of afferent nerve endings in injured esophageal mucosa is able to change the cough sensitivity only in patients with some subclinical pathological changes in the airways (12). For that reason we decided to assess the effect of intraesophageal administration of capsaicin in supramaximal concentration on citric- acid induced cough in guinea pigs with experimentally induced allergic disorder of the respiratory system.

Aim: The aim of the study was to assess the effect of intraesophageal administration of capsaicin (noxious stimulus activating nerve endings of C – fibres and subpopulation of Aδ nerve fibres) on citric acid induced cough in animals with experimental allergic rhinitis after passive sensitisation with ovalbumin and repeated sequence of active sensitisations with intranasal ovalbumin challenges, as well.

METHODS

Animals: All experiments were approved by Jessenius Faculty of Medicine Ethical Committee and followed the criteria of welfare of experimental animals, as well.

Animals (guinea pigs n = 16) (body weight 350-450 g) were housed in an approved animal house, maintained at room temperature 21-22^{o}C, humidity 60-70%, ventilation, 12-h light-dark cycle and free access to water and standard animal food. Male TRIK strain guinea pigs were obtained from the Department of Experimental Pharmacology, Slovak Academy of Science (Dobra Voda, Slovak Republic) and used after at least 1week adaptation period in the animal house. Guinea pigs were adapted to experimental conditions two times, by inhalation of nebulized saline in the plethysmographic box.

Ovalbumin sensitization: All animals (n = 16) were passively sensitized with ovalbumin (OA) (10 μg, Sigma) administered intraperitoneally together with aluminum hydroxide (100 mg) in saline (1 ml i.p) (13). Twenty-one days after successful sensitization was confirmed by skin prick test (intradermal injection of ovalbumin 25 μl of 200 $\mu g.ml^{-1}$) on the skin of the back. Only sensitised animals with marked erythema and oedema were involved in the study. Animals were used for experiments 7 days later.

Model of allergic rhinitis: Sensitized animals were used to develop a model of allergic rhinitis by repeated intranasal instillation of 0.015 ml of 0.5% OA into both nares via a thin catheter. These animals were repeatedly intranasally challenged with OA at 7-day intervals for 6 weeks. These challenges of antigen were followed by allergic nasal response (sneezing, nasal discharge and worsening of nasal breathing) and so this model of experimental allergic rhinitis was taken as confirmed.

Intraesophageal administration of stimulating substances: Animals (n=16) were placed into a plastic cylinder (a part of the body chamber of the plethysmographic box equipment (double chamber plethysmograph type 855, Hugo Sachs Elektronik, Germany). This allowed immobilization of animals.

After that, using a mouth opener device, thin portex catheter (external diameter 0.3 cm) with conducting wire was introduced into the esophagus of the animal. The catheter was tipped with cotton tampon and its end was positioned in the middle part of esophagus.

Capsaicin (Sigma, 1000 μM, 0.2 ml) was applied into the catheter in a manner that allowed absorption of the solutions used into the cotton tampon. Conducting wire was then introduced into the catheter and cotton tampon was disengaged from its tip by means of careful moving of the conducting wire. Then the catheter was pulled out from esophagus and mouth.

Induction of coughing: Awake guinea pigs were placed into the plastic cylinder (a part of plethysmographic box equipment) allowing immobilization of the animal. Then the animals were placed individually in a bodyplethysmograph (type 855, Hugo Sachs Electronic, Germany) (Fig.1) consisting of a head chamber and a body chamber. The opening between the head chamber and body chamber was equipped with a plastic collar lining around animal neck to prevent communication between the chambers. Appropriate collar size was chosen for each animal to prevent neck compression, which could cause airway obstruction and/or mechanical stimulation of the upper trachea and larynx.

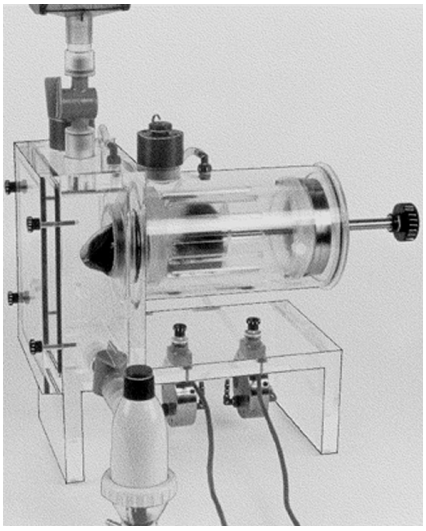

Fig. 1: Double chamber bodyplethysmograph (type 855, Hugo Sachs Elektronik, Germany) which is used in our department for induction of cough by administration of tussive aerosols into the front chamber of the plethysmograph.

To expose an animal to aerosol, the head chamber was connected to a nebulizer (Pari Provokation Test I, Menzel, Germany, manufacturer's specification: output 5 $l.min^{-1}$, particle mass median aerodynamic diameter 1.2 μm). A suction device adjusted to the same input 5 $l. min^{-1}$ was connected to the head chamber to maintain constant airflow through the chamber during aerosol administration. Respiratory changes in the airflow were measured using pneumotachograph (Godart, Germany) with Fleisch head (No. 1, Gould Godart Statham BV) connected to the head chamber and recorded directly with pen recorder (Multiscriptor Hellige, Germany).

Respiratory sounds including cough and sneezing were recorded with a microphone placed in the roof of the head chamber and connected to a preamplifier and loudspeaker. Pneumotachograph and tape recorder outputs were simultaneously recorded on a PC for off- line analysis.

Cough challenge was performed using inhalation of citric acid (CA) (Lachema, 0.3 M) for 1 minute. Cough was detected from the expiratory change of airflow interrupting basic respiratory pattern accompanied by a cough sound during 1 min exposure to CA and subsequent 2 minutes period.

Statistical analysis: Data for number of the cough efforts are expressed as a median and interquartil range. Statistical analysis of the samples was performed using a Kruskal – Wallis test. A probability value of $P< 0.05$ was considered as significant.

Experimental protocol

In all animals (n = 16) the control cough response to 0.3 M citric acid was determined. According reactivity the animals were divided into controls and experimental group.

After 7 days interval the animals of both groups were challenged with intranasal ovalbumin to induce rhinitis.

When the clear clinical signs of rhinitis were present (sneezing, nasal rubbing, crackles or discharge) controls were intraesophageally challenged with saline and experimental animals were intraesophageally challenged with supramaximal concentration of capsaicin.

The cough response to citric acid was determined just after the intraesophageal administration of capsaicin or saline.

These values (number of coughs) were compared to basal values obtained just in the beginning of the experimental protocol.

RESULTS

Model of allergic rhinitis

Clinical symptoms of rhinitis have occurred in all sensitized animals that were challenged intranasally with ovalbumin within 15 minutes after the challenge. These nasal symptoms involved sneezing, nasal discharge, nasal rubbing and nasal crackles. Some of the animals showed signs of laboured breathing - paradoxical movements of abdominal wall during inspiration (due to congestion of nasal mucosa).

Intraesophageal challenges

Intraesophageal challenges with both saline and capsaicin were well tolerated by animals. We did not notice any signs of esophageal irritation due to tampon (it means excessive salivation, vomitus or abnormal behaviour of the animals after the challenge). The animals had no problems with deglutition of standard food and water after the procedure.

Citric acid-induced cough

We have found that the number of cough efforts induced by inhalation of citric acid in controls was increased after the induction of rhinitis and intraesophageal administration of saline (med ± interquartil range) [4 (1.5-5) vs 6 (3.5-8), p = 0.05]. Very similar result was obtained in the group of experimental animals with rhinitis, which were intraesophageally challenged with supramaximal concentration of capsaicin (med ± interquartil range) [3 (2-4) vs 5 (5-6.5), p= 0.034].

This mentioned increase of the number of cough efforts is due to rhinitis, but on the other hand, we did not find any differences in the cough response that could be ascribed to intraesophageal administration of saline or capsaicin (p = 0.26) (Fig.2).

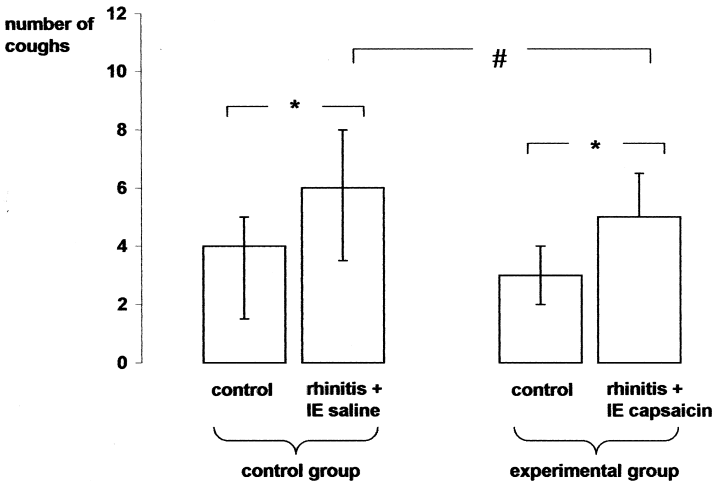

Fig. 2:
Changes of citric acid – induced cough intensity (expressed as a median and interquartil range of number of coughs) in guinea pigs with experimental allergic rhinitis during stimulation of esophageal mucosa. The increase of cough response in both groups is due to rhinitis, but there was found no difference in cough response that could be ascribed to esophageal administration of capsaicin (rhinitis + IE saline vs rhinitis + IE capsaicin)
* p< 0.05, # = non significant difference.

DISCUSSION

In our previous study we showed that the transient introduction of capsaicin into intact esophagus or esophagus injured by acid or alkaline pretreatment did not trigger cough or affect the citric acid-induced cough in the awake guinea pigs models. This esophageal stimulation does not influence either the specific airway resistance measured by Pennock`s method (2). These data indicate that acute localized stimulation of esophageal mucosal nerves is not sufficient to trigger and/or enhance cough in naive animals.

Our present study was based on the suggestion that stimulation of vagal afferents in the esophagus in patients suffering both from the GERD and the chronic cough could be sufficient to influence the reflex arc of coughing only in those patients with some subclinical changes in the airways (12). This suggestion could be also one of the possible explanations of our previous negative results in animals with injured esophagi, but intact respiratory system (2).

A model of allergic rhinitis in guinea pigs sensitized to ovalbumin was chosen for this purpose due to previous good experiences with this model as suitable for enhancement of citric acid-induced cough in guinea pigs. In this case the inflammatory process was located in the nasal cavity and probably close proximity of nasopharynx, however, it is well known that inflammatory process from the nasal cavity could be easily spread into the whole respiratory system (the larynx and the lower airways – the sites from which the cough could be elicited) via airways (post nasal dripping, microaspiration) (14) or via systemic circulation and subsequent homing of immune cells (15). We did not have a convincing evidence for the presence of inflammatory changes in the lower airways in guinea pigs suffering from experimentally induced allergic rhinitis. This caveat could be used for the interpretation of the negative results from these studies.

We have found that cough response in animals is enhanced, however, this increase of the cough intensity is due to rhinitis. This result is well known and was published before (14). But we did not find any differences in the cough response, which could be ascribed to intraesophageal administration of capsaicin.

Capsaicin, one of the most frequently used stimulants for sensory afferents, was chosen for esophageal vagal nerve endings. This substance acts via vanilloid receptor (TRVR1) on the nerve terminals. Morphological and neurophysiological studies indicate that only a proportion of the sensory nerve terminals in healthy esophageal mucosa can be accessed by diffusion form the esophageal lumen (16). To offset the barrier effect we introduced large concentration of lipofilic capsaicin (1000 μM, ≅ 1000 times the maximal effective concentration on the guinea pig TRPV1 receptor).

Our results indicate that this afferent input from the distal esophagus conducted to the nucleus of the solitary tract (nTs) was not sufficient to enhance cough in the guinea pig. NTs is the site of central projections of vagal afferents (including putative cough-mediating afferents) it is believed that esophageal afferents affect the activity of cough pathways neurons (11). Indeed, a mechanism of interaction between disparate afferent inputs (termed convergence) has been proposed in regulation of the bronchial tone (18, 19) and other C-fibres-mediated reflexes (20). However, although convergence would provide the simplest mechanism, the interaction between esophageal afferent input and cough pathways can take place at other central levels.

Another very interesting question is the chemosensitivity of the vagal afferent in the esophagus. Although chemo sensitivity of the esophageal vagal afferent was demonstrated in the guinea pigs, it was also found that in the dog, the esophagus appears to have scant vagal innervations, with a preponderance of SARs having a possible role in the mechanisms of deglutition. Moreover, there is a poor response to acid solutions and other irritants. These receptors have different properties than have vagal airway receptors do not appear to play an important role of the esophagus in response to chemical irritants (21).

In this relation, very important finding is that whereas the majority of cough associated with esophageal diseases is due to reflux, there is some recent experience suggesting that other esophageal diseases such as dysmotility and esophageal spasm may be equally important in the etiology of chronic cough rising from the upper gastrointestinal tract (21). It was suggested that

in these patients, cough may arise directly from receptors in muscle or in the submucsa stimulated by muscle contraction. There is also another factor that should be taken into consideration. Although ineffective esophageal motility (lack of mechanical protection against irritating substances and impaired transport of saliva) could be a pathogenetic factor in developing inflammatory changes in the esophagus, inflammatory changes in the esophageal wall could result into formation of esophageal strictures and dysmotility, as well (22).

The mechanisms responsible for coughing in patients with GERD are still not completely understood, but the close proximity of the trachea and the esophagus with the afferent nerve fibres of the vagus running between the two organs give the opportunity for multiple mechanisms to be relevant in the production of cough from esophageal disease (23).

Our results indicate that the acute guinea pig model is not suitable for studying the mechanisms of reflux–related chronic cough. The acute model probably cannot adequately mimic the changes occurring in esophagus and or neural pathways in patients with reflux– related chronic cough. The chronic exposure of esophagus to noxious components in refluxed material leads to functional and morphological changes in the esophageal sensory nerves. Indeed, the nerve density and as well as neuropeptide and nociceptive receptors expression by neural tissue is increased in the esophagi of patients with GER (24). In addition, chronic stimulation of esophageal sensory nerves may lead to changes in the properties and connectivity of central components of esophageal sensory pathways. For example, the expression of neurotransmitter substance P is regularly increased in sensory nerves supplying the injured tissues. Recent papers suggest that substance P acts as a volume neurotransmitter in the sites of central projections of vagal sensory nerves (i.e. nucleus of the solitary tract) and this effect may substantiate the interactions between esophageal and bronchopulmonary (including cough or bronchoconstriction) pathways (12). In any event, these plastic changes typically develop over long periods of time and are therefore inherently absent in the acute models (15). To further explore this question a chronic model of esophageal injury is needed.

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COGNITIVE EVOKED POTENTIALS IN PATIENTS FOLLOWING CONCUSSION

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Abstract

Background and purpose: Concussion is one of the most common neurological diagnosis that can lead to detecting the damage to cellular systems of the brain and long-term postconcussion symptoms. The aim of this study was to neuropsychologically and neurophysiologically examine the patients in acute period following concussion and to compare the results with the control group of volunteers.

Methods: Eleven patients and eleven controls were included in the study. They were neuropsychologically tested and underwent two standard auditory oddball ERP paradigms within 2 - 4 days after concussion.

Results: We found significant differences in all measured scores of reaction time, attentional and memory functions. There were no significant differences in measured variables of N2 and P3 wave.

Conclusion: Standard auditory oddball paradigm was insensitive to identify possible physiologic changes that are associated with cognitive, emotional and behavioral signs and symptoms soon after mild closed head injuries. In the future we plan to increase the number of patients and reexamine them in 3 to 6 months.

Keywords: concussion, mild traumatic brain injury, event-related potentials, cognitive evoked potentials, P300

INTRODUCTION

Concussion accounts for 70-80% of all head injuries and it is one of the most common neurological diagnosis. In the year 2003 the incidence of hospital-treated patients was about 250/100 000 in Slovakia and 310/100 000 in the Czech Republic (1,2). It was the 7-th most frequent reason for hospitalisation in the Slovak Republic. Concussions are mostly caused by motor-vehicle collisions and falls. The risk is approximately 2 times higher for males and higher in teenage and young adults (3).

Although concussions were considered as a temporary disruption of neurological functions without long-term effects, it is now understood that concussions may result in various transient and permanent damage to cellular systems of the brain that can lead to long-term postconcussion symptoms (4,5). Epidemiological studies have reported that 1 year after injury 15-50% of the patients declare postconcussive symptoms (6,7).

Standard methods used to assess the damage related to concussion are neurological examination, neuropsychological testing and structural imaging (CT, MR) with little emphasis on neurophysiological methods. In our study patients and controls were examined by two paradigms of event-related potentials (ERPs).

Cognitive ERPs belong to a wider group of neurophysiological methods. ERPs differ from evoked potentials while they are associated with cognitive processes such as attention, memory, executive functions and anticipation that follow processing information in primary sensory systems. They reveal activity of inner neuronal circles in the brain during performing specific tasks. Two components of the cognitive ERPs that deserve special attention with respect to mild brain trauma are the N200 (N2) and P300 (P3) responses. The generators of these waveforms have been attributed to medial temporal structures, hippocampus, thalamus, parietal lobe, temporo-parietal junction, frontal lobe (8). Given that concussions are often accompanied by difficulties in processing information, ERPs may be suited to detect such impairment particularly well.

METHODS

The aim of this study was to examine the patients in acute stage following concussion and to compare the results with control group of volunteers.

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Subjects: The group of patients consisted of n=11 subjects (7 men; M=31.45 years; SD= 11.69) who were suffered by concussion. Control group included n=11 volunteers (7 men; M=30.55 years; SD=12.2). Both groups did not differ in terms of age, sex and the level of education (Tab.1). All patients and controls were neurologically and neuropsychologically tested and underwent ERP, MRI and EEG examination within 2-4 days after the accident. The severity of the symptoms was assessed by Postconcussion Symptoms Scale (PCSS) (9) rating of 19 the most common postconcussion symptoms. The severity of each symptom was rated on a scale ranging from 0 (none) to 6 (severe).

Table 1. Population

	Patients	Controls	Difference /p/
Male/Female	7/4	7/4	
Age (Mean ± SD) years x	30.09 ± 11.12	29.18 ± 13.3	**0.58**
Education (Mean ± SD) years	13.18 ± 1.94	14.55 ± 2.38	**0.18**

Inclusion criteria: In our study concussion was defined as any blow to the head which results in temporary impairment of neurological functions such as unconsciousness up to 30 mins, confusion, disorientation, anterograde or retrograde amnesia not longer than 24 hours. Later on it can be accompanied by other postconcussive signs and symptoms such as headache, nausea, vomiting, drowsiness, dizziness, emotional and cognitive changes.

Exclusion criteria : We excluded subjects with history of chronic alcohol or drug abuse, previous traumatic brain injury, pre-existing neurological disorder (e.g. stroke, epilepsia, multiple sclerosis), psychiatric illnessess, arterial hypertension, diabetes mellitus and those under 18 years of age. Exclusion criteria were designed to minimise misleading effects of external influences on neuropsychological test scores, ERPs and structural brain imaging.

Neuropsychological testing: Neuropsychological testing used a battery of standard tests designed to identify abnormalities in reaction time, attention and memory. Memory was assessed by subtests of Wechsler Memory Scale, 3rd edition (10)- Logical Memory I&II, Faces I&II, Verbal Paired Associates I&II, Word list I&II, Letter-Number Sequencing. For our study we defined *Score of Working Memory and Attention* as the score of Letter-Number Sequencing subtest, *Score of Immediate Memory* as the sum of the scores of Logical Memory I, Faces I, Verbal Paired Associates I and Word List I subtests. *Score of Delayed Memory* is defined as the sum of scores of Logical Memory II, Faces II, Verbal Paired Associates II, Word List II subtests and *Score of Reaction Time* as the score of Disjunctive Reaction Time test (11).

Cognitive Event Related Potentials (ERP): Two standard auditory oddball paradigms were used with a series of tones containing 40 target and 160 non-target tones. A high tone of 2000 Hz served as a target stimulus and a low tone of 1000 Hz was the non-target stimulus. Each burst was presented at an intensity of 70 dB. The stimulus duration was 100 ms for both tones with an inter- stimulus interval of 1.25 s. During the task subjects were comfortably seated in a armchair. ERP responses were recorded from three midline scalp electrodes Fz, Cz, Pz applied according to the International 10-20 System. A reference electrode was attached to the left mastoid area and the ground electrode to the forehead. In the first paradigm the subject's instruction was as follows: „Try to relax and close your eyes. Count the number of higher tones. When the recording is finished I will ask you their number. " In the second paradigm the subjects pressed the button on each target tone and counted their number as well. We measured N2 and P3 latencies and amplitudes (baseline to peak), N2/P3 (peak to peak) amplitude in each paradigm and reaction time of pressing the button in second paradigm. Each paradigm was performed twice and mean values were calculated from both repetitions.

Statistics: We assumed that the data were not normally distributed and therefore the non-parametric Mann-Whitney U test was used for comparison between patiens and control groups in neuropsychological and ERPs variables. The significance level was set to alpha= 0.05. We used statistical program Past (website: <http://folk.uio.no/ohammer/past>).

RESULTS

PCCS and Neuropsychological testing (Table 2):

Patients declare more subjective complaints in PCCS than healthy volunteers in control group (p=0.03). Neuropsychological testing revealed signifficant differences in all measured scores of attentional and memory functions (A+WM: p=0.009; IM: p=0.009; DM:p=0.003). Differences in reaction time from both tasks (Table 4) were significant.

ERPs:

There were no significant differences in measured variables of N2 and P3 wave (Table 3,4).

Table 2. PCCS and Neuropsychological testing

Score	Patients (Mean ± SD)	Controls (Mean ± SD)	Difference p
PCCS	26.2 ± 18.78	9.5 ± 6.36	**0.03**
Attention and working memory	10.09 ± 2.74	13.2 ± 1.23	**0.009**
Immediate memory	130.27 ± 12.19	151.1 ± 17.64	**0.009**
Delayed memory	140.82 ± 18.28	165.6 ± 10.25	**0.003**
Reaction time	31.9 ± 13.0	48.67 ± 10.24	**0.015**

Table 3. ERP – first paradigm

	Patients (Mean ± SD)	Controls (Mean ± SD)	Difference p
N200 lat (ms)	289.26 ± 32.81	274.25 ± 29.91	**0.28**
N200 ampl (μV)	10.78 ± 3.89	10.88 ± 3.11	**0.97**
P300 lat (ms)	396.82 ± 26.87	402.92 ± 21.59	**0.46**
P300 ampl (μV)	8.01 ± 3.05	8.38 ± 2.37	**0.65**
Ampl N2/P3 (μV)	18.78 ± 5.53	19.26 ± 4.54	**1**

Table 4. ERP – second paradigm

	Patients (Mean ± SD)	Controls (Mean ± SD)	Difference p
N200 lat (ms)	278.17 ± 21.97	274.43 ± 26.34	**0.55**
N200 ampl (μV)	9.09 ± 3.52	9.64 ± 3.5	**0.46**
P300 lat (ms)	390.91 ± 29.64	406.9 ± 29.19	**0.23**
P300 ampl (μV)	7.01 ± 3.67	7.81 ± 2.08	**0.65**
Ampl N2/P3 (μV)	16.1 ± 5.25	17.45 ± 3.99	**0.60**
Reaction time (ms)	385.82 ± 90.9	289.9 ± 52.24	**0.01**

DISCUSSION

Traditionally, early ERP components, such as N100 and P200 waves have been considered to be a manifestation of primarily sensory processing in auditory cortex or auditory association

areas, whereas later ERP components including N2 and P3 waves have been associated with cognitive processes. Shape of N2 wave appears to depend on retaining and comparing memory traces of stimuli, a process that leads to categorization of the current stimulus and selection of the appropriate response. Longer latency and smaller amplitude are associated with fewer cognitive resources involved in stimulus discrimination (12). P3 latency is normally considered to reflect speed of stimulus evaluation and categorization. P3 amplitude is a manifestation of attentional allocation to a stimulus, subjective significance and stimulus probability (13). N2-P3 potential have been used to assess the severity of severe closed head injuries, as well as the recovery period (14,15,16,17). In our study of patients with mild brain injury we found no significant differences in N2 and P3 latencies and amplitudes, despite the positive neuropsychological findings. Prolongation of patients' reaction times (RT) both in Disjunctive Reaction Time test and in the second paradigm did not produce a prolongation of N2-P3 latencies. The reason for this is, that stimulus evaluation time which contribute to N2-P3 latency was not affected and RT prolongation was due to slowness of response execution time (18). It has been proposed, that patients who sustained a head injury are unsure about responding. We suppose, that the insignificant reduction of mean P3 latency in the patients group is probably due to a small size of the sample. Moreover, neuropsychologically proven impairment of attention has no correlation in the attenuation of the P3 amplitude. The P3 amplitude is usually reduced when the processing required for the stimulus evaluation is more complex due to difficult task (19) or limited capacity of attentional resources directed to the stimulus evaluation (20,21). Both standard paradigms we used, were not difficult enough to exhaust attentional capacity and therefore did not lead to a significant amplitude reduction. And for this reason more complex auditory oddball paradigms such as Dichotic Listening task (22), Three-tone task (23), Duration or Duration with Distraction tasks (19,22,24) and also Speech Evoked task (25), Contingent Negative Variation paradigm (19,24) seem to be more suitable to reveal an attentional limitation.

There are inconsistencies in results between studies that use standard auditory oddball paradigm soon after the concussive injury. Our results are similar to those of Werner and Vanderzant (26). They found, that standard paradigm was insensitive in quantifying the possible physiologic changes that are associated with cognitive, emotional and behavioral symptoms after mild closed head injuries. On the other hand Pratap-Chand et al. (27) studied a group of 20 controls and 20 patients with acute mild head injury and found significant abnormalities of P300 latency and amplitude. This discrepancy with our results can be due to fact that variables such as age and level of education were probably not fully considered. Two other studies (28,29) have demonstrated significant prolongation of the P3 latency, when measured in the immediate postconcussion period. In this period patients were still confused and had problems with encoding information to memory (which result in postraumatic amnesia-PTA). During the period of recovery from PTA, P3 latency decreased. However, all our patients were already oriented at the time of ERP examination. We suppose that difference of the postinjury time evaluation is the main reason for differences between the results in our studies.

We realize that a small number of patients in our case-control study, is the main weak point of it. We plan to test more subjects and then evaluate differences in ERP variables of patients with and without MR abnormalities.

There are a few studies of standard auditory ERPs, in which patients were not examinated soon after head injury, but several months and years after. They reveal inconsistent results. In studies of Sangal and Sangal (30), Gaetz and Weinberg (8) no significant differences were found in N2/P3 parameters. In another study, P3 latency and amplitude abnormalities were found in a group of mild to moderate head injury subjects (31). Another two studies reported a reduction in P3 amplitude in patients several years after mild head injury (19,24). For better comparisons with these studies, all our patients and controls will be tested once more in 3 to 6 months time. If we find any abnormalities in parameters N2/P3, despite their absence soon after the injury, we suppose, they could be caused by subtle organic changes, that can develop during the weeks following the concussion (4,5,32).

We found that standard auditory oddball ERP paradigm was insensitive to identify possible physiological changes that are associated with cognitive, emotional and behavioral signs and

symptoms soon after mild closed head injuries. In the future we plan to increase the number of patients and reexamine them in 3 to 6 months.

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BLOOD SERUM ALUMINIUM CONTENT IN GENERAL POPULATION COMPARED TO DIALYSED PATIENTS

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Abstract

Objective: The goal of this work was to examine the concentrations of aluminium (Al) in the blood serum of children and adults (who during their life were not professionally exposed to aluminium) in Martin Region Slovakia, to find out the average concentrations of aluminium in the serum of general population of this region. These values were compared to the concentrations of Al in the serum of adult patients of Nephro - Dialysis Centre of Martin suffering from chronic renal insufficiency.

Methods: The authors examinated blood sera of 27 adults and 24 children and 53 blood sera of the dialysed patients (DP). The used analytical method was the atomic absorption spectroscopy in graphite furnace (GFAAS) with detection limit of samples 0.1 - 5 $\mu mol.l^{-1}$ and temperature 2500 °C.

Results: The values of serum aluminium of adults were 0.521 ± 0.05 $\mu mol.l^{-1}$ (x ± SE) and of children were 0.320 ± 0.04 μmol.l-1 (x ± SE). The measured concentrations of Al in the blood serum of dialysed patients were 0.530 ± 0.03 $\mu mol.l^{-1}$ in year 2002 and 0.916 ± 0.08 $\mu mol.l^{-1}$ in 2004. In comparison to the control group of adults the values of aluminium in the serum of the dialysed patients in 2002 were not higher. However, the examination of the same dialysed patients two years after (in 2004) revealed a significant increase of serum aluminium.

Conclusion: Although the concentrations of serum aluminium were safe, the regular monitoring should be maintained also in the future.

Key words: aluminium, non-occupational exposure, dialysed patients, serum

INTRODUCTION

Aluminium (Al) is the most abundant metal and the third most abundant element, after oxygen (O_2) and silicon (Si), in the earth's crust. This element is very reactive (in compounds as Al^{3+}) and it is never found as free metal in the nature. It is found in compounds with other elements, most frequently with oxygen, silicon, and fluorine. These chemical compounds are commonly found in soil, rocks and clays. These are the natural forms of aluminium rather than the silvery metal. The metal is obtained from aluminium-containing minerals, primarily bauxite. Small amounts of aluminium are even found in water in dissolved or ionic form. The most frequenty found ionic forms of aluminium are complexes formed with hydroxyl (OH^-) ions (1).

We are most familiar with aluminium in beverage cans, pots and pans, aeroplanes, roofing, and foil. Aluminium metal is light in weight and silvery-white in appearance. Since pure aluminium is very soft, aluminium is often mixed with small amounts of other metals. These aluminium compounds are used in many diverse and important industrial applications. The world industrial production is about 29 million tons per year mainly for automobile industry. Powdered aluminium metal is often used in explosives and fireworks. It is also found in the consumer products such as antacids, astringents, buffered aspirin, food additives, and antiperspirants.

The way of its intake into the food chain is from Al contaminated air or earth's surface layer into the raining water, then into drinking water and finally into the food plants by bio concentrate. The human body is always exposed to some aluminium either through gastrointestinal tract, or by breathing air, or sometimes even through skin (1).

Aluminium levels in urban and industrial areas can vary between 0.4 - 10 $ng.m^{-3}$. The max-

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imum permitted concentration of metal aluminium in working area during occupational exposure in Slovakia is 1.5 $mg.m^{-3}$ (2). The amount of aluminium people inhale by breathing during the day is much lower than that they consume in food. They may breathe in higher levels of aluminium in the form of dust if they live in areas where the air is dusty, where aluminium is mined or processed into aluminium metal (1).

People generally consume very little aluminium from drinking water (3). Drinking water is sometimes treated with aluminium salts and generally the levels of aluminium in water are increasing. The maximum permitted concentration of aluminium in drinking water in Slovakia is 0.200 $mg.l^{-1}$ (4). People are exposed to aluminium also through some cosmetics and pharmaceuticals. The amount of aluminium ingested in antacids on the base of Al is as much as 200 milligram per tablet. Aluminium occurs naturally in many different kinds of food. Eating large amounts of processed food containing aluminium additives, cooking acid food in aluminium pots, or taking aluminium-containing drugs are the most common ways how families may be exposed to high levels of aluminium. But, on the other hand, it has been proved that aluminium cooking utensils, aluminium foil, antiperspirants, antacids, and other aluminium products are generally safe. The human body contains small amount of aluminium, but its biological significance has not been clarified yet. Aluminium is regarded as a non-toxic element for the current population. Despite of that the acid rains and the use of aluminium in industry can cause a dramatic increase of the amount of aluminium in the ecosystem. Therefore, the level of aluminium in population will be increasing.

The daily intake of Al is 2 mg for babies and 7 - 14 mg for adults (5). The gastrointestinal tract is relatively impermeable to aluminium. The normal absorption is only about 2%. Al is absorbed by a mechanism related to calcium. Gastric acidity and oral citrate favour absorption, H_2-blockers reduce absorption. Transferrin is the primary protein binder and an aluminium carrier in the plasma, where 80% is protein bound and 20% is free or compound in small molecules. Most aluminium leaves body quickly in faeces. The small amount of aluminium that does enter the bloodstream leaves in the urine. Very little amount can enter body through the skin and lungs (3). Al is cumulated mainly in the brain and bones and during professional exposition largely in lungs.

The patients with chronic renal failure are exposed to more aluminium than the healthy population. Their daily intake of Al is not only from food and drinking water but also from Al-antacids such as phosphate binders and Al-containing dialysis fluids. At the beginning of the 1980s dialysed patients (DP) were heavily overloaded with Al (6). At present time the mentioned factors have been eliminated: elimination of Al content in dialysis concentrate by producer, purification of water for preparing dialysis solution, application of antacids on the base of calcium carbonate, not on the base of Al. The removal of Al by dialysis is not easy because almost 85 – 90% is protein-bound aluminium, thus only a small amount of Al (10 - 15%) is diffusible and ultrafiltratable (6). Elevated aluminium levels have been the cause of various disorders, including dialysis encephalopathy or dementia, Al-induced bone disease and microcytic anaemia (7). Dialysis dementia can arise after three to seven years of haemodialysis treatment. Speech disorders precede dementia and convulsions. The death in this case has been reported as sudden cardiac arrest usually associated with acute pulmonary oedema (8). Aluminium contributes to various forms of renal osteodystrophy, because absorbed Al quickly leaves the serum and accumulates in the bone, where it is protected from excretion (9).

Several deaths have been reported in 1960s after occupational exposure to a finely powdered metallic aluminium used in paints, explosives, and fireworks (10). It should be noted that changes in production technology, the use of breathing masks and controls of the dust levels in factories have resulted in decreased occupational exposures to finely powdered aluminium. Factory workers who breathe large amounts of aluminium dust can have lung problems, such as coughing. Pulmonary fibrosis is the most frequently reported respiratory effect observed by workers after long term exposure to fine aluminium dust, aluminium oxide, or bauxite. However, the reports prove the fibrinogen potential of aluminium. In some of the cases, the fibrosis was attributed to concomitant exposure to other dusts with content of silicon oxide (11). For example, pulmonary fibrosis has been observed in a number of bauxite workers. By these workers, it is very

likely that there was simultaneous exposure to silica and that this latter was the causative agent rather than the aluminium (12, 13).

There is a relationship between Alzheimer's disease and accumulation of Al in brain tissue. Though we do not know for certain whether aluminium accumulation is a result of this disease or its cause (14).

The amount of aluminium can be measured in the blood (serum), urine, dialysis fluid or cerebrospinal fluid. These measured values are important for the diagnosis of intoxication, monitoring of aluminium exposed persons (industry) and for patients with chronic renal insufficiency .

METHODS

The goal of this study was to detect the concentration of Al in the blood serum. Four groups of samples were examined: DP 02 - dialysed patients from Martin in 2002 (n =38), DP 04 - dialysed patients from Martin in 2004 (n =36), CA - control adults (n =27) and CCH - control children (n =24). The samples of serum in volume 1 ml were prepared from 5 ml of native blood. All samples were stored in refrigerator at 5 °C and analysed within three days. The analytical method which was used was atomic absorption spectroscopy in graphite furnace (GFAAS) with detection limit of sample 0.1 - 5 $\mu mol.l^{-1}$ and the atomise temperature 2500 °C. The atomic absorption spectrophotometer AAS Varian Spectr. AA 30 P was used with graphite furnace GTA - 96. All measurements were performed at the Clinic of Occupational Medicine and Toxicology, Faculty Hospital Martin.

Results of the examinations were processed by mathematical and statistical methods. Arithmetic mean values (x), standard error of the arithmetic mean (±SE) and standard deviations (±SD) were calculated. Groups were compared by using Student's t-test. The data were processed by means of the Stargraphics programme.

A group of 24 children living in Martin was examined. This group was used as a control group. Serum samples contained of 0.5 ml of serum which remained from other examinations. We supposed that the concentration of Al in body and afterwards in the blood increases by age, therefore we expected the concentration to be low.

RESULTS

All results are presented in tables and figures. Table 1 shows the basic characteristics of the examined groups - the number of people in the group and their mean age. Figure 1 shows the concentrations of Al in the serum of the studied groups from Martin.

Table 1 Basic characteristics of the examination groups - dialysis patients in years 2002 and 2004, the control groups of adults and children: the number of examinated, the mean age and age range

	DP-02 Dialysed patients 2002	**DP-04** Dialysed patients 2004	**CA** Control adults	**CCH** Control children
N	38	36	27	24
mean age in years	59.01	60.95	36.21	5.78
± SD	15.09	15.66	11.20	2.77
± SE	2.45	2.61	2.16	0.57
Minimal	20	22	20	1
Maximal	88	90	66	10

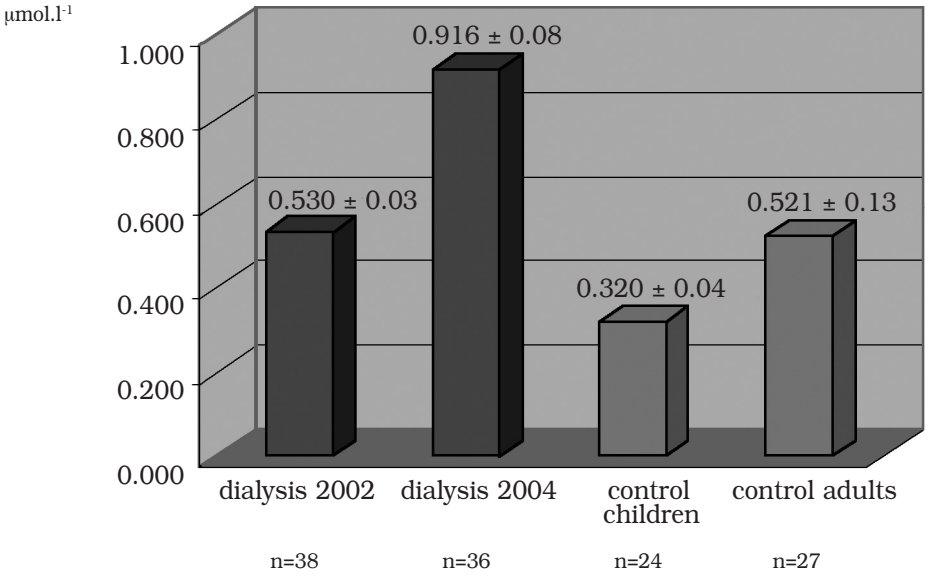

Fig. 1 The average concentration of Al ($\bar{x}$ ± SE μmol.l^{-1}) in the serum of the examined groups: dialysed patients in years 2002 and 2004, adults and children from Martin

The children had low concentrations of Al in the serum ($\bar{x}$ ± SE, 0.320 ± 0.04 μmol.l^{-1}). We tried to prove that the content of Al in the blood serum increases by age. Correlation coefficient for relation between age and concentration of Al in the serum was + 0.411 for the control group of adults (CA) and + 0.083 for the control of children (CCH) - the non significant correlation coefficients. Therefore, these measurements do not confirm relation between Al concentration in the serum and the age, but it is necessary to examine a larger group of people in the future. In comparison to the control group of adults, dialysed patients did not have increased concentrations of serum aluminium in 2002. These two groups of DP (DP - 02 and DP - 04) were not compared, because the groups did not have the same members.

Seventy percent of the group members DP-02 and DP-04 (n=21) consisted of the same people. We compared their concentrations after two years and we could see the increased values of Al concentrations in the serum of dialysis patients after two years of dialysis programme (Table. 2, Fig. 2). The values of concentration of Al in the serum were significantly increased, $p<0.001$ in 2004.

Table 2. Age characteristics of the compared groups of the same dialysed patients from dialysis centre in Martin repeatedly examined in 2002 and in 2004 for concentrations of Al in the serum

	DP MT-02 Dialysed patients Martin 2002	**DP MT- 04** Dialysed patients Martin 2004
N	21	21
mean age in years	55.04	57.04
± SD	16.4	16.4
± SE	3.58	3.58
Minimal	20	22
maximal	88	90

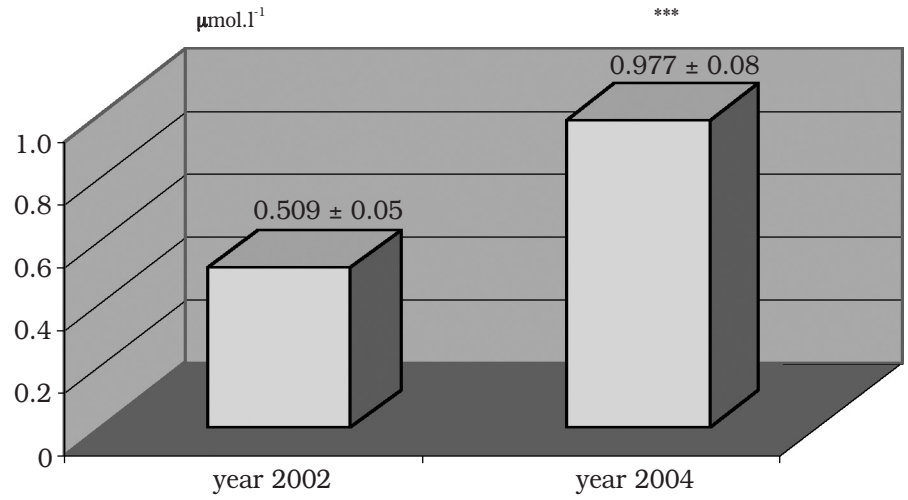

Fig. 2. The average concentration of Al ($\bar{x}$ ± SE μmol.l^{-1}) in the serum of dialysis patients from Martin, years 2002 and 2004, n=21, *** p<0.001

DISCUSSION

The recent measurements of aluminium concentrations in the serum of population without occupational exposure revealed the normal background levels 0.320 - 0.521 μmol.l^{-1}. The threshold limit value for aluminium in the blood serum in Slovakia is not defined. The extent of normal values is determined by the concentrations of Al in the serum of the general population and this interval is used as the background. Commission of the European Community (CEC) recommends reference normal value for individuals with normal renal function < 10 μg.l^{-1} (= 0.371μmol.l^{-1} - 15). Other authors present reference Al value in the serum: 0.110 μmol.l^{-1} (16). Because of the ubiquitous nature of aluminium contamination, we assume, that there is a direct relationship between Al in the environment of the Martin Region and the content of Al in the body. Moreover, the content of Al in drinking water is very important, although our experimental data do not provide consistent evidence.

The average Al serum concentrations in children was lower than the concentrations in adults. We did not prove that the serum concentration of Al in the population increases with age. Taking into consideration life style, treatments in the past, the eating habits, some samples of children showed higher concentration of Al in serum than in the adults. We could not to compare the average concentrations between the control group of children and the group of dialysied children, because the second one did not have enough members.

Individuals with chronic renal failure requiring long-term haemodialysis treatment are a group within the general population that may be exposed to greater than background levels of Al. Although aluminium is already not present in the dialysate in the recent ten years we reported increased serum Al concentrations in dialysed patients comparing to the control group. The concentrations of Al in the serum of dialysed patients after two years increased significantly, but these concentrations were safe. The objective of the study from Spain was to analyse the changes in the aluminium content in dialysis fluid and the effect on serum aluminium in 17 dialysis centres in Spain in 8 years. The concentrations of serum were decreasing from year to year onto the value 25.7 μg.l^{-1} (= 0.953μmol.l^{-1} - 6). Sulkova described a patient, where aluminium was the cause of various disorders, including dialysis encephalopathy and aluminium-induced bone disease proved by a bone biopsy, and microcytic anaemia.This concentration of Al in the serum before administration of desferrioxamine was 460 μg.l^{-1} (= 17.049 μmol.l^{-1} - 17). Commission of the European Community (CEC) recommends reference desirable value by chronic renal failure

patients < 60 μg.l^{-1} (= 2.224 μmol.l^{-1}) and points out the value > 200 μg.l^{-1} (= 7.413 μmol.l^{-1}) where an urgent action is required, because high risk of toxicity exists in all cases (15).

Aluminium balance in haemodialysis depends mainly on the gradient of diffusible aluminium, on the type of dialysis membranes, on their surface and thickness and also on many other factors, such as the pH of the dialysate. Among all these factors, undoubtedly the most important is the concentration of aluminium in dialysis fluids (18). Maximum allowed concentration for dialysis fluid is 30 μg.l^{-1} (= 1.112 μmol.l^{-1}) - CEC recommendation (15).

The increased of values Al in serum of DP (groups DP MT - 02 vs DP MT - 04) after two years of haemodialysis is the evidence that the regular annual monitoring of the serum concentration of Al of dialysed patients remains necessary.

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