Advances in Experimental Medicine and Biology 1023
Neuroscience and Respiration

Mieczyslaw Pokorski *Editor*

Pulmonary Disorders and Therapy

Springer

Advances in Experimental Medicine and Biology

Neuroscience and Respiration

Volume 1023

Subseries Editor
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Pulmonary Disorders and Therapy

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ISSN 0065-2598 ISSN 2214-8019 (electronic)
Advances in Experimental Medicine and Biology
ISBN 978-3-319-73702-7 ISBN 978-3-319-73703-4 (eBook)
https://doi.org/10.1007/978-3-319-73703-4

Library of Congress Control Number: 2017964514

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Preface

The book series Neuroscience and Respiration presents contributions by expert researchers and clinicians in the multidisciplinary areas of medical research and clinical practice. Particular attention is focused on pulmonary disorders as the respiratory tract is up front at the first line of defense for organisms against pathogens and environmental or other sources of toxic or disease-causing effects. The articles provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of diseases and disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with biomedicine at both the molecular and functional levels and with the interactive relationship between respiration and other neurobiological systems, such as cardiovascular function, immunogenicity, endocrinology and humoral regulation, and the mind-to-body connection. The authors focus on modern diagnostic techniques and leading-edge therapeutic concepts, methodologies, and innovative treatments. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical, data-driven options to manage patients are considered. New research is presented regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children is also discussed.

Body functions, including lung ventilation and its regulation, are ultimately driven by the brain. However, neuropsychological aspects of disorders are still mostly a matter of conjecture. After decades of misunderstanding and neglect, emotions have been rediscovered as a powerful modifier or even the probable cause of various somatic disorders. Today, the link between stress and health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches, which can decrease stress, can play a major role in disease therapy.

Neuromolecular and carcinogenetic aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to disorders, are also tackled.

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Clinical advances stemming from molecular and biochemical research are but possible if research findings are translated into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All this cannot be achieved without a multidisciplinary, collaborative, bench-to-bedside approach involving both researchers and clinicians. The role of science in shaping medical knowledge and transforming it into practical care is undeniable.

Concerning respiratory disorders, their societal and economic burden has been on the rise worldwide, leading to disabilities and shortening of life-span. Chronic obstructive pulmonary disease (COPD) alone causes more than three million deaths globally each year.

Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the articles published in this series will assume a leading position as a source of information on interdisciplinary medical research advancements, addressing the needs of medical professionals and allied health-care workers, and become a source of reference and inspiration for future research ideas.

I would like to express my deep gratitude to Paul Roos, Tanja Koppejan, and Cynthia Kroonen of Springer Nature NL for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Mieczyslaw Pokorski

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 1–10
DOI 10.1007/5584_2017_48
© Springer International Publishing Switzerland 2017
Published online: 21 July 2017

Acute and Chronic Effects of Oral Erdosteine on Ciliary Beat Frequency, Cough Sensitivity and Airway Reactivity

L. Pappová, I. Kazimierová, M. Jošková, M. Šutovská, and S. Fraňová

Abstract

Erdosteine as a mucolytic agent that decreases mucus viscosity and facilitates mucus expulsion from the airways by cough or ciliary movement. Our objective was to determine whether erdosteine can directly contribute to mucus clearance. We addressed the issue by monitoring acute and chronic effects of erdosteine on ciliary beat frequency (CBF), cough sensitivity, and airway smooth muscle reactivity. The experiments were performed in healthy guinea pigs. Erdosteine (10 mg/kg) was administrated orally in a single dose or daily through 7 days. The cough reflex and specific airway resistance were evaluated *in vivo*. The CBF in tracheal brushed samples and the contractile response of tracheal smooth muscle stripes to bronchoconstrictive mediators were evaluated *in vitro*.

We found that neither acute nor chronic erdosteine treatment had a significant effect on cough sensitivity and airway reactivity. However, in the *vitro* condition, erdosteine increased CBF and reduced tracheal smooth muscle contractility; the effects were more pronounced after chronic treatment. We conclude that erdosteine may directly contribute to mucus clearance by CBF stimulation. Although erdosteine has no effect on cough reflex sensitivity, its mild bronchodilator and mucolytic properties may promote effective cough.

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Keywords
Airway reactivity • Airway smooth muscles • Ciliary beat frequency • Cough sensitivity • Erdosteine • Mucus clearance

1 Introduction

Mucus production and its continuous expulsion from the airways, through the mucociliary or cough clearance, represent an important innate defense mechanism preventing the lungs, despite their being continuously exposed to different environmental insults, against injury (Fahy and Dickey 2010). However, excessive production of airway mucus and subsequent changes in its biophysical properties may hamper mucus clearance, leading to its accumulation in the respiratory tract. Under this condition, mucus becomes rather harmful than protective (Van der Schans 2007). Indeed, airway mucus congestion is a feature of a number of severe respiratory diseases. There is growing evidence that mucus retention contributes to disease symptomatology and it also can significantly affect the disease pathology (Rogers 2007). Thus, optimal treatment of obstructive airway diseases should focus not only on suppression of the underlying inflammation, but also should address the poor mucus clearance. Current pharmacologic options provide a variety of mucoactive drugs, including oral mucolytics such N-acetylcysteine or erdosteine (Balsamo et al. 2010). The term mucolytic refers to a compound with a free sulfhydryl group that mediates the reduction in mucous viscosity, which is supposed to result in facilitated mucus expulsion (King 2006). However, despite the long-standing presence of these agents in clinical practice, their effectiveness in hyper-secretory airway diseases has not been clearly established, which likely reflects the uncertainties about the mechanisms of the drugs action (Rogers 2007). While, it may have seemed easy to characterize the mucolytic effect, it is much more difficult to define this effect in terms of mucus clearance, as mucus is not the unique single component involved in the defense mechanism (Davies and Calverley 2010).

Modulation of mucus clearance by the mucolytic agent erdosteine is the main focus of this experimental study. Erdosteine is a homocysteine analogue with two blocked sulfhydryl groups, which are released following the first-pass metabolism providing three active metabolites (Dechant and Noble 1996). Although all three metabolites contain a free sulfhydryl group, one active most metabolite (Met 1) seems responsible for the majority of pharmacodynamic properties of erdosteine (Dal Negro 2008). Beside the positive effect on mucus rheology, Met 1 has a significant free radical scavenging and antioxidant activity (Moretti and Marchioni 2007; Braga et al. 2000), reduces the bacterial adhesiveness to human epithelium (Braga et al. 1999), and exerts some anti-inflammatory activity suppressing chemotactic cytokines (Park et al. 2016). In the pathologic condition, these effects may indirectly improve mucus clearance. Therefore, in the present study we seek to determine whether erdosteine would have the ability to directly modulate mucus clearance by altering the following: 1/ ciliary beat frequency (CBF), an essential determinant of mucociliary clearance (King 2006), and 2/ cough reflex sensitivity, an important back-up mechanism of mucus clearance, when mucociliary clearance falters (Munkholm and Mortensen 2014). We addressed the issue in the physiological conditions in an attempt to avoid the influence of other possible erdosteine co-activities. Further, we also addressed the effect of erdosteine on airway reactivity, as it has been demonstrated that some cysteine analogues such N-acetylcysteine, may cause bronchospasm and thus worsen the airway obstruction (Reinero et al. 2011).

2 Methods

The study was approved by the Institutional Ethics Committee of the Jessenius Faculty of Medicine (permission IRB 00005636). All experimental procedures were performed in accordance with the Slovak and European Community regulations for the use of laboratory animals and guidelines on animal welfare (decision No. 1249/2013). Healthy, adult male TRIK-strain guinea pigs, weighing 300–400 g, were purchased from the accredited breeding facility of the Department of Experimental Pharmacology, Slovak Academy of Sciences, Dobrá Voda, Slovakia. The animals were housed under the control conditions with free access to food and water.

Erdosteine was purchased from Abcam Biochemicals (Cambridge, UK). Reference drugs (salbutamol and codeine) and the other chemicals such as citric acid and bronchonstrictive mediators (histamine and acetylcholine), were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). Erdosteine and codeine were dissolved in water for injection and all other substances were dissolved in 0.9% saline.

2.1 Experimental Groups

The animals were randomly divided into experimental groups consisting of 10 animals each. The animals assigned to the erdosteine group were treated with erdosteine in a dose of 10 mg/kg, administered as a peroral water solution. The erdosteine treatment was performed either acutely as a single dose or chronically, once daily through 7 days. Guinea pigs in the control group received only the water vehicle (1 ml/kg) and those in the positive control groups were treated with the reference bronchodilator and ciliostimulatory drug salbutamol (4 mmol/l) by way of the inhalation and the antitussive oral drug codeine (10 mg/kg). Salbutamol solution was aerosolized in a PARI jet nebulizer (Paul Ritzau; Pati-Werk GmbH, Starnberg, Germany, output 5 1/s, particles median diameter 1.2 μm) and was delivered to the head chamber of double body box (HSE type 855; Hugo Sachs Electronik, March, Germany), where the animals were placed in.

Both acute and chronic erdosteine effects on cough reflex and airway reactivity *in vivo* were assessed 2 h after the last erdosteine administration. Subsequently, animals were sacrificed by cervical dislocation. The upper part of the trachea was used to collect ciliary samples for determining the ciliary kinematics, and the lower part was exploited to prepare tracheal smooth muscle stripes used for monitoring the contractile response to bronchoconstrictor mediators.

2.2 Evaluation of Cough Reflex

Conscious guinea pigs were placed individually in a double chamber body box. The cough reflex was provoked chemically by exposure to citric acid aerosol lasting for 3 min, during which the number of cough efforts were counted. Citric acid solution, at a concentration of 0.3 mol/l was aerosolized by a PARI jet nebulizer. A sudden enhancement of expiratory flow during coughing was detected by a pneumotachograph connected to the nasal chamber of the body box. Cough effort was defined according to the ERS guidelines as a sudden PC-recordable enhancement in expiratory airflow, accompanied by the characteristic cough sound and movement that were evaluated by two trained observers and additionally verified with the video recordings (Morice et al. 2007).

2.3 Evaluation of Airway Smooth Muscle Reactivity *In Vivo*

Airway smooth muscle reactivity *in vivo* was evaluated in conscious animals placed in the double chamber body box. The bronchodilator potential of erdosteine was evaluated on the basis of specific airway resistance (sRaw), calculated according to the method of Pennock et al.

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(1979) from the phase shift between the nasal and thoracic respiratory flows using the HSE Pulmodyn Pennock respiratory software (Hugo Sachs Electronik; March, Germany). Airflow changes were provoked according to method of Kazimierová et al. (2015). Briefly, animals were exposed for 30 s to histamine aerosol at a concentration 10^{-6} mol/l. The exposure was followed by a 1-min period of fresh air insufflation into the nasal chamber and then the sRaw measurement was taken.

2.4 Evaluation of Airway Smooth Muscle Reactivity *In Vitro*

Airway smooth muscle reactivity *in vitro* was tested in isolated tracheal smooth muscle stripes using Experimetria IS02 eight-channel modular tissue bath system with software (Experimetria Ltd., Budapest, Hungary). Two tracheal smooth muscle stipes were taken from each animal. The stripes were placed into a 20-ml bath chamber filled with Krebs-Henseleit buffer consisting of NaCl 112.9, KCl 4.7, $CaCl_2$ 2.8, $MgSO_4$ 0.5, $NaHCO_3$ 24.9, and glucose 11.1 (all mmol/l) at 36.5 ± 0.5 °C, pH 7.5 ± 0.1, and being continuously saturated with 95% O_2 and 5% CO_2. Initially, tissue stripes were adjusted to 4 g tension during a 30-min loading phase, followed by readjustment to the baseline tension of 2 g for the following 30 min. Throughout both phases, tracheal stripes were washed at 10 min intervals. Thereafter, the stripes were exposed to cumulative doses of acetylcholine (10^{-8} – 10^{-3} mol/l) and histamine (10^{-8} – 10^{-3} mol/l) with a continuous recording of contractile response (Franova et al. 2016).

2.5 Evaluation of Ciliary Beat Frequency *In Vitro*

After the animals had been sacrificed, a small window was dissected in a precisely cleaned upper part of the trachea to access the epithelial layer for brushing out the ciliated cells. The acquired material was resuspended on a heated microscope slide in a drop of warm saline solution (36.5 °C ± 0.5) and was coverslipped. The stripes of ciliated epithelium with beating cilia were selected using an inverted phase contrast microscope (Zeiss Aixo vert. A1; Carl Zeiss AG, Göttingen, Germany) and the cilia beating was recorded with a high speed video camera (Basler A504kc; Adept Turnkey Pty Ltd., Brookvale, Australia) with the frame rate of 256–512 *per* second. Short video clips of cilia beating regions, approx. 10 video clips *per* sample, were analyzed with Labwiew™ software. Ciliary regions of interest (ROI), intensity variations in selected ROI, and the intensity variance curves were generated. The curves were further analyzed with the fast Fourier transformation algorithm (FFT). Fourier spectrum of each intensity variance curve was then equal to the frequency spectrum of cilia beating in each ROI. For every ROI, the median of ciliary beat frequency (CBF) was calculated and used as an evaluation parameter. The final value of CBF, expressed in Hz, was the average of ten median values obtained from each specimen.

2.6 Statistical Analysis

All results are expressed as means ± SE. Statistical analysis was performed using one-way analysis of variance ANOVA. A p-value of less than 0.05 was taken as a threshold for statistical significance.

3 Results

3.1 Cough Reflex

Changes in cough reflex, evaluated *in vivo,* were assessed before drug administration to obtain the baseline level in the control group. The erdosteine's effect on cough reflex was recorded for 2 h after a single dose and 2 h after the final dose of 7-day treatment. The effect was compared with that of the reference antitussive drug codeine, administrated in like manner. The evaluation was based on the number of cough efforts

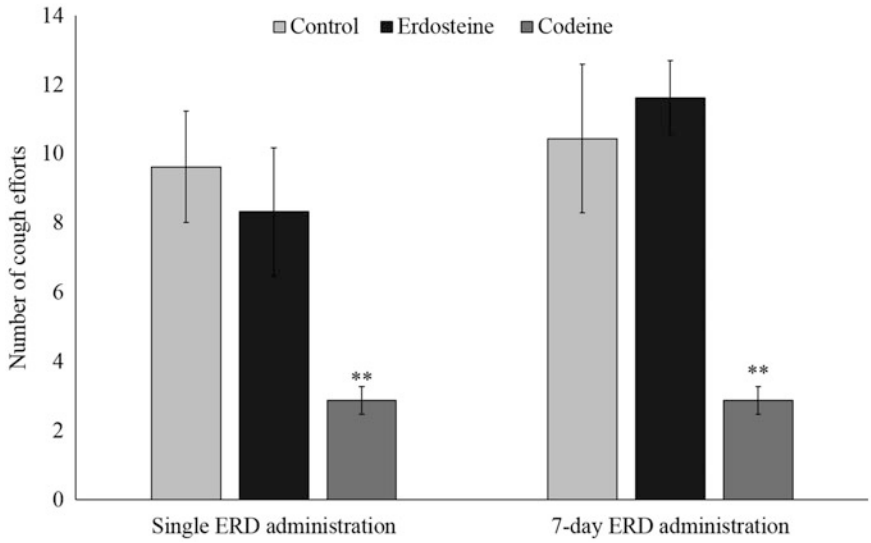

Fig. 1 Citric acid-induced cough reflex, expressed as the number of cough efforts, after a single dose and 7-day oral erdosteine (ERD) treatment of healthy adult guinea pigs compared to naïve (control) and codeine (reference drug)-treated animals. Data are means ±SE; $n = 10$ in each group; $^{**}p < 0.01$ *vs.* control

evoked by citric acid inhalation used. Neither acute nor chronic erdosteine treatment exerted a significant effect on cough sensitivity, whereas codeine significantly suppressed cough efforts (Fig. 1).

3.2 Airway Reactivity *In Vivo*

The airway contractile response *in vivo* was provoked by histamine inhalation and the evaluation was based on the measurement of sRaw before and 2 h after erdosteine treatment. Both single and 7-day erdosteine treatments tended to decrease sRaw. However, the bronchodilator tendency of erdosteine failed to reach statistical significance as opposed to the evident bronchorelaxing activity of the reference drug salbutamol (Fig. 2).

3.3 Airway Reactivity *In Vitro*

The bronchodilator potential of erdosteine *in vitro* was assessed by recording changes in the amplitude of tracheal smooth muscle contraction in response to cumulative doses of acetylcholine and histamine. After both acute and chronic erdosteine treatment, the bronchoconstrictor response to higher concentrations of the mediators was attenuated compared with the control baseline level. The strongest decline in contraction amplitude of tracheal smooth muscle was recorded in response to the highest concentrations of histamine of 10^{-4} and 10^{-3} mol/l after 7-day erdosteine treatment. This decline was akin to the anti-contractile effect of the the reference drug salbutamol (Fig. 3a). The effects of cumulative doses of acetylcholine on tracheal smoth uscle contractility were less pronounced (Fig. 3b). The declines from the control baseline level in muscle contractility in reponse to the highest acetylcholine concentrations of 10^{-5} and 10^{-3} mol/l also were similar after 7-day erdosteine and salbutamol treatment, but failed to achieve statistical significance.

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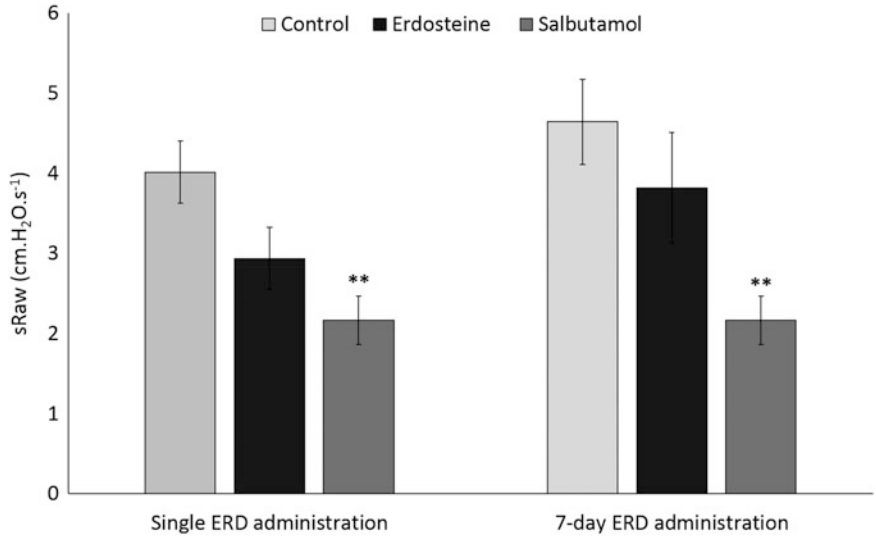

Fig. 2 Histamine induced airway reactivity *in vivo*, expressed as specific airway resistance (sRaw), after a single dose and 7-day oral erdosteine (ERD) treatment of healthy adult guinea pigs compared to naïve (control) and salbutamol (reference drug)-treated animals. Data are means ±SE; $n = 10$ in each group; $^{**}p < 0.01$ *vs.* control

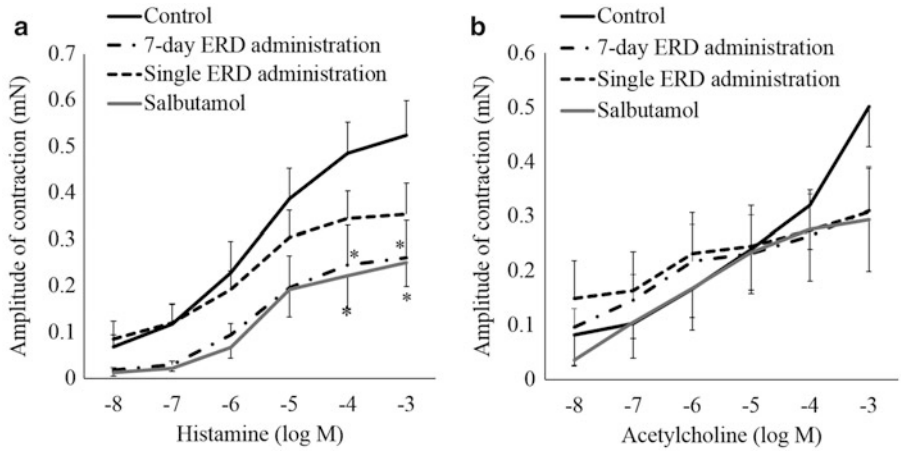

Fig. 3 Tracheal smooth muscle contractile responses *in vitro* to cumulative doses of histamine (**a**) and acetylcholine (**b**), expressed as the amplitude of contraction, after a single dose and 7-day oral erdosteine (ERD) treatment of healthy adult guinea pigs compared to naïve (control) and salbutamol (reference drug)-treated animals. Data are means ±SE; $n = 10$ in each group; $^{*}p < 0.05$ *vs.* control

3.4 Ciliary Beat Frequency (CBF)

The erdosteine effect on ciliary movement was evaluated *in vitro* by recording changes in CBF. Both acute and chronic erdosteine treatment caused a significant increase in ciliary beating, with a greater ciliostimulatory activity observed after the chronic treatment. However, the reference drug salbutamol showed the most distinct CBF stimulation (Fig. 4).

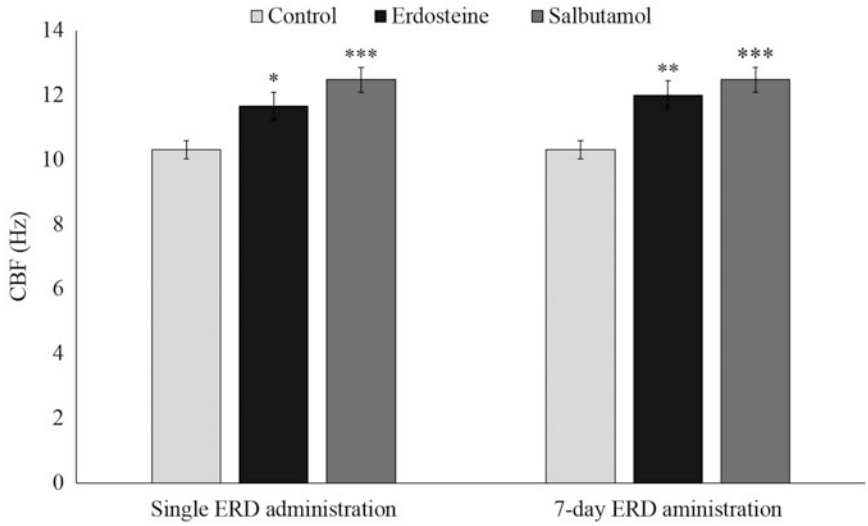

Fig. 4 Ciliary beat frequency (CBF) after a single dose and 7-day oral erdosteine (ERD) treatment of healthy adult guinea pigs compared to naïve (control) and salbutamol (reference drug)-treated animals. Data are means ±SE; $n = 10$ in each group; $^{*}p < 0.05$ *vs.* control; $^{**}p < 0.01$ *vs.* control; and $^{***}p < 0.001$ *vs.* control

4 Discussion

The airway mucus layer may be defined as a gel primarily composed of water and high molecular glycoproteins mucins, which gives mucus its characteristic viscoelastic properties (Thornton and Sheehan 2004). In the normal condition, low viscosity and elasticity of mucus facilitate its removal from the airway by means of ciliary beating (Fahy and Dickey 2010). In pathological conditions, particularly characterized by inflammation, a highly elastic and viscous mucus formed uncouples the effective interaction between cilia and the mucus layer, resulting in defective mucociliary clearance and consequently mucus accumulation (Seagrave et al. 2012). The changes in mucus composition are associated with a higher content of disulphide cross-links between mucin glycoproteins (Yuan et al. 2015). Erdosteine as a mucolytic agent that disrupts the mucin polymer network by reducing disulphide bounds. The consequent decrease in mucus elasticity and viscosity is expected to increase mucociliary transport rate (Hosoe et al. 1999). The CBF is a key factor influencing the rate of mucus transport (Braiman and Priel 2008). The mechanism of ciliary beating regulation is the subject of a number of recent studies that have revealed the influence of many receptors, second messengers, and ion channels (Joskova et al. 2016; Workman and Cohen 2014; Salathe 2007). It also has been found that inflammatory mediators exert an inhibitory effect on ciliary function (Pappová et al. 2016; Thomas et al. 2010). Several studies have previously demonstrated the increased mucociliary clearance after erdosteine treatment (Dal Negro 2008; Olivieri et al. 1991). However, to our knowledge, no study has attempted to evaluate a direct effect on ciliary movement of erdosteine. In the present study, the ciliomodulatory activity of erdosteine was evaluated after a single dose and chronic 7-day long erdosteine treatment, and the erdosteine influence was compared with that of salbutamol, used as a reference drug in this study. We found a significant increase in CBF already present after a single dose of erdosteine, with a pronounced enhancement of the effect after chronic erdosteine treatment; the latter effect achieved the potency close to that present after salbutamol treatment. Since the experiment was performed in the physiological condition, we surmise that we observed a direct stimulatory action on ciliary cells of erdosteine, which

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could hardly be mediated by indirect actions of erdosteine such as its possible anti-inflammatory or antioxidant activity.

A combination of mucolytic and ciliostimulatory effects of erdosteine may facilitate the mucociliary transport rate by increasing mucus expulsion from the airways and reducing airway obstruction. As mucus, congested and retained in the airways, irritates cough receptors sensitive to mechanical stimuli, evoking cough (Polverino et al. 2012). Actually, cough is the primary symptom of mucus retention and it partially compensates for the impaired mucociliary clearance (Munkholm and Mortensen 2014). It follows that the enhancement in mucociliary clearance may result in decreased cough sensitivity. However, the efficacy of clearance also depends on the properties of mucus layer that may be more viscous but not adhesive to the airway epithelium (Rubin 2014). Erdosteine as a mucolytic agent that by hydrolysing the polymeric structure of the outer secretory surface may relieve the secretory content from the epithelium (Rubin 2010), and thus promote effective cough; reducing the cough frequency with time (Dicpinigaitis et al. 2014). Nonetheless, mucolytics are not regarded as very effective in ameliorating cough in respiratory pathologies (Bolser 2006), which may be due to the fact that cough, beside mechanical stimuli, also depends on chemical triggers, including inflammatory mediators (Polverino et al. 2012). Concerning erdosteine, several experimental studies have pointed to the drug's possible antitussive activity (Dal Negro 2008; Hosoe et al. 1999). In the present study, we found no effect of erdosteine in the physiological condition, either in single or chronic dosing, on cough reflex evoked by citric acid inhalation compared to both naïve animals and the reference antitussive opioid drug codeine. The attenuation of citric acid-induced cough by erdosteine observed in other studies (Dicpinigaitis et al. 2014; Dal Negro 2008) might rather have to do with the drug's antioxidant and anti-inflammatory properties.

Another pathologic feature of airway mucus congestion is a significant airflow limitation, which worsens with airway narrowing due to bronchoconstriction (Rogers 2004). Thus, bronchodilator activity of a mucolytic agent may be of benefit. We addressed this issue in the present study by examining the effect of erdosteine on airway reactivity, assessed in both *in vivo* and *in vitro* conditions and compared with the reference bronchodilator drug salbutamol. A statistically relevant bronchodilator activity of erdosteine was recorded only in the *in vitro* condition, observed as the ability of chronic erdosteine treatment to reverse the tracheal muscle contraction induced by histamine. This effect was akin to the attenuated contractile response observed in the salbutamol-treated group. However, neither did acute nor chronic erdosteine treatment manage to significantly decrease acetylcholine-mediated tracheal muscle contraction, although a tendency to lessen the contractile response was seen on par with salbutamol. Overall, these findings suggest that erdosteine confers a week bronchodilator activity. One randomized placebo controlled study, carried by Dal Negro et al. (2008), has revealed that erdosteine increases the bronchodilating activity of β_2-adrenergic drugs; the activity attributed by the authors to the erdosteine-mediated protection of adrenergic receptors against desensitization caused by free radicals.

We conclude that erdosteine is a multifactorial mucolytic drug that increases the mucus transport by several mechanisms. Erdosteine's influence on mucus consistency, decreasing mucus viscosity and elasticity, and enhancing ciliary beat frequency underlie the drug's efficacy in mucociliary clearance. The mucolytic-mediated increase in mucociliary clearance may also attenuate cough, even though erdosteine, by itself, does not seem to have the inherent antitussive activity. In addition, bronchodilating potential of erdosteine also improves the ciliary clearance by increasing the expiratory airflow.

Acknowledgements Supported by grants: APVV 0305-12, VEGA 1/0165/14 a MZ 2012/35- UK, CERK II, UK/62/2016; by the project Biomedical Center Martin – ITMS: 26220220187 entitled 'We support research activities in Slovakia', co-financed from EU sources.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 11–27
DOI 10.1007/5584_2017_51
© Springer International Publishing Switzerland 2017
Published online: 6 July 2017

Inhalation Exposure to PM-Bound Polycyclic Aromatic Hydrocarbons Released from Barbecue Grills Powered by Gas, Lump Charcoal, and Charcoal Briquettes

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Abstract

The present study seeks to define the possible cancer risk arising from the inhalation exposure to particle (PM)-bound polycyclic aromatic hydrocarbons (PAHs) present in barbecue emission gases and to compare the risk depending on the type of fuel used for grill powering. Three types of fuel were compared: liquid propane gas, lump charcoal, and charcoal briquettes. $PM_{2.5}$ and $PM_{2.5–100}$ were collected during grilling. Subsequently, 16 PAHs congeners were extracted from the PM samples and measured quantitatively using gas chromatography. The content of PM-bound PAHs was used to calculate PAHs deposition in the respiratory tract using the multiple path particle dosimetry model. Finally, a probabilistic risk model was developed to assess the incremental lifetime cancer risk (ILCR) faced by people exposed to PAHs. We found a distinctly greater PAHs formation in case of grills powered by charcoal briquettes. The summary concentration of PAHs (Σ16PAH) ranged from <0.002 μg/m^3 (gas grill) to 21.52 μg/m^3 (grill powered by briquettes). Daily exposure

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of a grill operator, while grilling meat, to $PM_{2.5}$-bound PAHs, adjusted to benzo[a]pyrene toxicity equivalent (BaP_{eq}), was 326.9, 401.6, and 0.04 ng/d for lump charcoal, charcoal briquettes, and gas powered grill, respectively. Exposure to PAHs emitted from charcoal briquettes was four orders of magnitude greater than that for gas grill. The ILCR followed a log-normal distribution, with a geometric mean of 8.38×10^{-5} for exposure to $PM_{2.5}$-bound PAHs emitted from gas grills unloaded with food and as high as 8.68×10^{-1} for the grills loaded with food over charcoal briquettes. The estimated cancer risk for people who would inhale barbecue particles for 5 h a day, 40 days a year exceeds the acceptable level set by the U.S. Environmental Protection Agency. We conclude that the type of heat source used for grilling influences the PM-bound PAHs formation. The greatest concentration of PAHs is generated when grilling over charcoal briquettes. Loading grills with food generates conspicuously more PAHs emissions. Traditional grilling poses cancer risk much above the acceptable limit, as opposed to much less risk involving gas powered grills.

Keywords
Barbecue • Grill • Inhalation exposure • Particulate matter • Pollution • Polycyclic aromatic hydrocarbons

1 Introduction

According to the data gathered by Hearth, Patio and Barbecue Association (HPBA 2015), more than 72% of all US households own a grill, with almost half grilling at least 1–2 times per week during summer months. Likewise, barbecuing is in the vogue in Poland, one of the 26 countries belonging to the World Barbecue Association. According to the Millward Brown report of 2012, 66% of Poles grill each sunny weekend in the spring and summer. Beyond the excellent taste, grilled food brings certain health risks, associated with both intake of toxic compounds generated during the heat processing of food and exposure to barbecue-emitted gases and in particular to fine ambient particles (Chen et al. 2012). Smoke and soot particles produced during grilling, loaded with carcinogenic and mutagenic compounds such as heterocyclic amines and polycyclic aromatic hydrocarbons (PAHs) (Aaslyng et al. 2013), slowly waft into the air and penetrate into the lungs (Taner et al. 2013). While heterocyclic amines (HCAs) are mostly formed as an effect of meat carbonization, PAHs are created when fat drips melt on charcoal. The hazard from particulate (PM)-bound PAHs is attributed to their mass distribution regarding the particle size. Although the precise mechanism of PM-bound PAHs deposition in the respiratory tract is unknown, it is considered that minute particles loaded with those compounds are responsible for the adverse health effects.

Barbecue fumes aggravate asthma and other respiratory disorders (Adewole et al. 2013). The elderly suffering from chronic obstructive pulmonary disease (de Batlle et al. 2012; Jiang et al. 2008; Jiang et al. 2007; Varraso et al. 2007), bronchitis (Rajpandey 1984), and children are particularly sensitive to barbecue smoke and soot. Some studies demonstrate that PAHs inhaled with ambient particles might be hazardous for fetal development (Choi and Perrera 2012). Seow et al. (2000) have found that the exposure to emission gases from meat cooking might increase the risk of lung cancer among

Chinese women. A formation of HCAs and PAHs varies by food type, cooking method, and doneness level (rare, medium, or well done) (Jägerstad and Skog 2005). Fuel types such as wood, coal, gas, and electricity can produce marked differences in the concentrations of PAHs during grilling. A study by Ciemniak (2007) has demonstrated that the amount of benzo(a)pyrene, expressed in μg/kg, in chicken meat grilled by the traditional charcoal method to the well done level is greater by 67% compared to that obtained during the electric grilling. In case of chicken skin, the difference reached 355% by weight. Similar results have been published by Food and Environmental Hygiene Department (FEHD 2004) showing that charcoal grilling of 'Siu Mei' gives rise to the highest amount of PAHs when compared with gas grilling or electric oven roasting. A number of studies have demonstrated the occurrence of genotoxic and carcinogenic PAHs in foods grilled by different methods (El-Saeid 2010; Farhadian et al. 2010; Jägerstad and Skog 2005; Sundararajan et al. 1999). Most of them are focused on the dietary exposure to hydrocarbons formed during the pyrolysis of fat dripping on heat source from the food product and then migrating into the human body. On the other hand, few studies have investigated the level of inhalation exposure to PAHs generated in the course of grilling, depending on the type of heat source. Among various variables which affect the inhalation exposure from grilling, the type of barbecue installation and of heat source are probably the two most important. Different experimental studies indicate that traditional charcoal and briquettes generate larger quantities of PAHs, compared to electric or gas grills (Farhadian et al. 2010). In terms of PAHs emission, lump charcoal made from natural substrates such as beech, birch, hard maple, hickory, or oak hard woods is less hazardous compared to charcoal briquettes (Kim Oanh et al. 1999). Unlike the pure lump charcoal, charcoal briquettes are made from a mixture of different combustible components (e.g., sawdust, peat fines, coal, wood, or straw) compressed with some additives (oil, limestone, or petroleum products) that bind them together and facilitate consistent burning, but also favor the PAHs release (Kushwaha et al. 1985). In Canada, for instance, there are restrictions connected with selling charcoal briquettes (Canada 2009). Nonetheless, due to the briquettes' uniform shape, the ability of lighting easily and maintaining a steady temperature for a long period of time, they are still most often used for the grilling purpose worldwide.

Given the public health significance of PAHs emission, the goals of the present study were to determine the amount of these compounds in barbecue gases, to compare the level of inhalation exposure to PM-bound PAHs depending on type of fuel used for grill powering, and to estimate the deposition ratio of barbecue originating PAHs in different regions of the respiratory tract.

2 Methods

2.1 Sample Collection

The study was approved by a Research Board of Warsaw Technology University in Poland. It was performed in a private garden located in a suburban area of Warsaw. The area was characterized by a relatively high density of single-family houses with the allotting gardens. A dense settlement of houses and gardens contributed to the development of specific local communities, willingly participating in barbecue meetings. Three kinds of outdoor grill installations were tested: gas grill heated by liquid propane, traditional grill fueled by lump charcoal, and by charcoal briquettes. The traditional grill grate's surface was 1661 cm^2 and that of gas grills was 2742 cm^2. Two possible scenarios were tested: grill unloaded and loaded with food. When loaded, each grill's grate was covered by the same mass and kind of food. For this purpose, standard barbecue packs containing pork meat (sausages and steaks) were used. The grilling took place at windless outdoor weather, at 25 °C, on August 10–13, 2016. For traditional grills, the grilling time was 110 min and included four steps: 1/ initial lighting of the grill consisting of setting up the fire and burning the

fuel until the disappearance of flames and obtaining a uniform, bright streaks of smoke (20 min); 2/ glowing and slowly fuel burning (20 min); 3/ cooking time (60 min); and fire extinguishing (10 min). For gas grilling, the whole process took 80 min and consisted of three steps: 1/ lighting up the gas and maintaining constant temperature of 180-190 °C (20 min); 2/ cooking time (1 h); and turning off gas (few sec). Assuming that the grill operator did not stand in the immediate reach of the highly smoldering, burning fuel nor was he near the grill when it was extinguished, stages I, II, and IV were taken as the baseline level for the sampling procedure, while the food processing stage was treated as the vital exposure time. During grilling, each piece of food was turned every 10 min. The monitoring system was switched on 40 and 20 min after the charcoal and gas, respectively, was ignited at the time when the cooking started.

Two PM fractions were collected for each of the three grill installation at the same time: $PM_{2.5–100}$ (particles with aerodynamic diameter between 2.5 and 100 μm) and $PM_{2.5}$ (particles not greater than 2.5 μm). Two GilAir PLUS aspirators (Gilian, Sensidyne LP; St. Petersburg, FL) were located above each installation at 1.5 m height, simulating inhalation conditions by a grill operator, one adjusted to sample $PM_{2.5}$ and the second for $PM_{2.5–100}$. The size selective sampling heads were used to measure both fractions. PM was collected on the quartz fiber filters (QMA, ø25 mm; Whatman, GE Healthcare Bio-Sciences Corp; Piscataway, NJ). Prior to sampling, filters were baked at 600 °C for at least 6 h to remove any traces of organics. The filters were weighed and put into Petri dishes, wrapped tightly in the aluminum foil and stored at −18 °C until analysis. The sample mass was determined by weighing the substrates before and after exposure (MYA 5.3Y.F microbalance; resolution 1 μg; Radwag; Radom, Poland). Samples were conditioned in the weighing room (air humidity of 45 ± 5%, air temp. 20 ± 2 °C) for 48 h before each weighing. After mass determination, filters were directed for PAHs analyses. The grilling experiment was performed in three replicates and the measurements obtained were averaged (Table 1).

2.2 Sample Extraction and Instrumental Analysis

Before extraction, the pre-cleaned filter samples were spiked with the surrogate standard from Cambridge Isotope Laboratories (Tewksbury, MA), containing a mixture of seven deuterated PAHs: acenaphthylene (D_8), benzo[a]pyrene (D_{12}), benzo[g,h,i]perylene (D_{12}), fluoranthene (D_{10}), naphthalene (D_8), phenanthrene (D_{10}), and pyrene (D_{10}). Each of PM filters was next sonicated with 20 cm^3 of dichloromethane (Sigma-Aldrich; Poznan, Poland). The extraction was carried out twice with a constant flow of cooling water for 30 min. The extracts obtained were filtered and concentrated to 1 cm^3. All samples were analyzed with a Shimadzu gas chromatograph coupled to a mass spectrometer (GCMS-2010 Plus) and were processed with the Shimadzu GCMS solution software (Shimadzu; Kyoto, Japan). A ZB-5MS capillary column (30 m × 0.25 mm i.d. with a 0.25 μm film thickness) was used for chromatographic separation. The column temperature was programmed from the initial 80 °C (held for 2 min) to a stepwise increase by 10 °C *per* min to 280 °C (held for 15 min). All samples were automatically injected (1 mm^3 each) at a constant injector's temperature of 250 °C. Ultrahigh purity helium at a flow rate of 1.5 cm^3 *per* min was used as a carrier gas. The ion source temperature was set at 250 °C. The mass selective detection was conducted in the electron impact mode. Mass spectra were acquired in the selected ion monitoring mode with the electron impact energy of 70 eV. In the test samples, the following 16 PAH compounds, present in the US Emergency Planning and Community (EPA list), were monitored: naphthalene, acenaphthylene, acenaphthene, fluorine, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno[1,2,3-cd]

Table 1 Average concentration of 16 PAHs and ΣPAHs emitted with PM from three kinds of barbecue installations (μg/m^3)

No.	PAH's symbol	Rings	$PM_{2.5}$						$PM_{2.5–100}$					
			G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]
			Without food			Loaded with food			Without food			Loaded with food		
1	NAP	2	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.4700	0.9800
2	AcPy	3	<0.0001	<0.0001	<0.0001	<0.0001	4.4000	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.3200	0.3800
3	Acp	3	0.0005	0.0008	0.0008	0.0008	0.0008	0.0008	0.0003	0.0003	0.0003	0.0005	1.5800	0.0003
4	Flu	3	0.0006	0.0007	0.0008	0.0006	1.5400	0.0006	0.0002	0.0003	0.7500	0.0002	0.2300	0.5200
5	PA	3	0.0009	0.0010	0.0013	0.0001	0.1300	1.1500	0.0001	0.0002	0.3000	0.0002	0.0600	0.1300
6	Ant	3	0.0003	0.0002	0.0002	0.0003	0.6800	0.0003	<0.0001	<0.0001	<0.0001	<0.0001	0.3600	0.7300
7	FL	4	0.0001	0.0003	0.0003	0.0004	0.4300	1.2100	<0.0001	<0.0001	<0.0001	<0.0001	0.7100	0.7300
8	Pyr	4	0.0003	0.0003	0.0002	0.0004	0.5200	1.2100	0.0002	0.0002	1.6900	0.0003	1.0400	0.8300
9	BaA	4	<0.0001	<0.0001	<0.0001	<0.0001	2.2900	5.0800	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
10	CHR	4	0.0004	0.0004	5.6000	0.0006	1.5000	3.3200	0.0003	0.0003	0.0003	0.0005	0.0006	0.0007
11	BbF	5	0.0006	0.0005	0.0007	0.0006	3.4600	5.5800	0.0002	0.0003	0.0002	0.0002	0.0005	0.0067
12	BkF	5	0.0003	0.0005	0.0006	0.000	0.0007	0.0007	0.0002	0.0001	0.0002	0.0002	0.0003	0.0004
13	BaP	5	0.0003	0.0003	0.0004	0.0003	0.0004	0.0005	0.0005	0.0005	0.0003	0.0006	0.0007	0.0006
14	IND	6	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	1.3100
15	DBA	5	<0.0001	<0.0001	<0.0001	<0.0001	3.8700	3.9700	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
16	BghiP	6	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	1.6400
Σ PAHs (μg/m^3)			0.004	0.005	5.605	0.005	18.822	21.523	0.002	0.002	2.741	0.003	4.772	7.259
BaP_{eq} (ng/m^3)			0.408	0.410	56.535	0.460	4474.287	5073.304	0.511	0.519	3.111	0.663	8.821	159.546

[a]G – grill powered by gas
[b]LC – grill powered by lump charcoal
[c]B – grill powered by charcoal briquettes

Table 2 The list of PAHs, their carcinogenic potency, toxicity, and quality control and quality assurance results

PAH's name	Symbol	Rings	CAS[a]	Genotoxicity[b]	IARC classification[c]	TEF[d]	Recovery %
Naphthalene	NAP	2	91-20-3	Negative	2B	0.001	167.7
Acenaphthylene	AcPy	3	208-96-8	Questionable	Not evaluated	0.001	105.6
Acenaphthene	Acp	3	83-32-9	Questionable	3	0.001	103.4
Fluorene	Flu	3	86-73-7	Negative	3	0.001	88.3
Phenanthrene	PA	3	85-01-8	Questionable	3	0.001	100.6
Anthracene	Ant	3	120-12-7	Negative	3	0.010	86.3
Fluoranthene	FL	4	206-44-0	Positive	3	0.001	96.0
Pyrene	Pyr	4	129-00-0	Questionable	3	0.001	100.3
Benz[a]anthracene	BaA	4	56-55-3	Positive	2B	0.100	104.5
Chrysene	CHR	4	219-01-9	Positive	2B	0.010	90.1
Benzo[b]fluoranthene	BbF	5	205-99-2	Positive	2B	0.100	94.6
Benzo[k]fluoranthene	BkF	5	207-08-9	Positive	2B	0.100	91.3
Benzo[a]pyrene	BaP	5	50-32-8	Positive	1	1.000	88.0
Indeno[1,2,3-cd] pyrene	IND	6	193-39-5	Positive	2B	0.100	82.3
Dibenz[a,h]anthracene	DBA	5	53-70-3	Positive	2A	1.000	106.2
Benzo[ghi]perylene	BghiP	6	191-24-2	Positive	3	0.010	89.6

[a]CAS – a unique numerical identifier assigned by Chemical Abstracts Service
[b]WHO Classification (1998) International program on chemical safety. Environmental health criteria 202, selected non-heterocyclic and polycyclic aromatic hydrocarbons (http://www.inchem.org/documents/ehc/ehc/ehc202.htm)
[c]IARC Classification (2015) of the International Agency for Research on Cancer: group 1 – the agent is carcinogenic to humans; group 2A – the agent is probably carcinogenic to humans; group 2B – the agent is possibly carcinogenic to humans; group 3 – the agent is not classifiable concerning human carcinogenicity (http://monographs.iarc.fr/ENG/Classification/)
[d]TEF – toxic equivalency factor for selected polycyclic aromatic hydrocarbons (PAH) based on the toxicity of benzo[a] pyrene (Nisbet and LaGoy 1992)

pyrene, dibenz[a,h]anthracene, and benzo[ghi] perylene (Table 2).

2.3 Quality Control

The mean extraction recovery for PAHs depends on the individual substance. The average recovery values obtained for target standards in the matrix-spiked blank samples were in the range of 82–106% as shown in Table 2. The limit of determination was 0.0001 μg/cubic meter (m^3) for all PAHs.

2.4 Inhalation Exposure and Health Risk Assessment

The inhalation exposure to PAH mixture was adjusted to benzo[a]pyrene toxicity equivalent (BaP_{eq}) using the toxicity equivalent factor for each compound (Eq. 1):

$$BaP_{eq} = \sum_{i=1}^{n} C \cdot TEF_i \quad (1)$$

Thus, BaP_{eq} (carcinogenic equivalent; ng/m^3) was calculated by multiplying the concentrations of each PAH compound by its cancer specific toxicity equivalent factor, relative to BaP carcinogenicity. The BaP_{eq} for the sum of 16 - non-volatile PAHs was as follows:

$$\begin{aligned}(BaP_{eq})\sum 16PAH = {} & [NAP] \times 0.001 \\ & + [AcPy] \times 0.001 + [Acp] \times 0.001 \\ & + [Flu] \times 0.001 + [PA] \times 0.001 \\ & + [Ant] \times 0.01 + [FL] \times 0.001 \\ & + [Pyr] \times 0.001 + [BaA] \times 0.1 \\ & + [CHR] \times 0.01 + [BbF] \times 0.1 \\ & + [BkF] \times 0.1 + [BaP] \times 1 \\ & + [IND] \times 0.1 + [DBA] \times 1 \\ & + [BghiP] \times 0.01\end{aligned}$$

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The health risk resulting from inhalation exposure to PAHs emitted from the barbecue was calculated following the US EPA recommendations (RAGS 1989). The exposure scenario was based on a few assumptions concerning the exposure time and frequency, and human physiology. In Poland, the barbecue season starts in May and lasts until the end of September, covering mainly the weekends. We assumed that the maximum frequency which the consumer stays with close to barbecue emitted gases is 40 days/year (5 months × 4 weekends). Thus, exposure frequency was approximated by lower (20 days/year) and upper (40 day/year) limit values. Following the method of Wu et al. (2015), we assumed that exposure duration to the PM-bound PAHs by grill operator/consumer over the lifetime is 53 years. The IQS96 Omnibus (1996) online study conducted in a cohort of 800 Polish individuals aged 15–50, using the Computer-Assisted Web Interview – Real Time Sampling (CAWI – RTS) methodology, indicates that 39% of grill consumers take part in barbecue meetings for 2–3 h, 35% for 4–5 h, and that 15% such meetings takes more than 5 h a day. Only do 11% of Polish people barbecue for 1 h or less. The average exposure time to barbecue fumes in the present study was thus modeled as the minimum/maximum range of 1/5 h. Considering that the grill operators are usually adults (age range from 20 to 70 years), the breathing rate under light activity conditions was chosen as 22.8 m^3/days (US EPA 2011). Finally, PAHs concentrations were modeled on the basis of the distribution of probability density function. Since PM data generally follow log-normal distribution (Widziewicz and Loska 2016), it was assumed that the same holds true for the PM-bound PAHs. However, the log-normality testing was not performed in this study, due to a small dataset.

The daily level of inhalation exposure (IE; μg/day) of PAHs for grill operator/consumer was calculated as a product of BaP_{eq} concentration (μg/m^3), inhalation rate (IR; m^3/h), and daily exposure time span (t; hour/day) (Eq. 2):

$$IE = BaP_{eq} \cdot IR \cdot t \tag{2}$$

The ILCR posed by exposure to PAHs emitted from barbecues was computed following Eq. 3. The calculation of health risk involved the multiplication of inhaled exposure level (IE) by carcinogenic slope factor for BaP_{eq} according to Eq. 2. Some variables in the risk equation were modeled as the distribution of probability density function (PDF) (Table 3).

$$ILCR = \frac{CSF_{inh} \cdot IE \cdot EF \cdot ED \cdot cf}{BW \cdot AT} \tag{3}$$

where: *ILCR* – incremental lifetime cancer risk of BaP_{eq} exposure (dimensionless); C*SF* – inhalation cancer slope factor for BaP (mg × kg/day), which was used to evaluate the relationship between the concentration of a certain PAH compound and the corresponding cancer risk. For this study, *SF* was derived from a study of Chen and Liao (2006) – a geometric mean of 3.14 (mg×kg/day) ± geometric standard deviation of 1.80. The proper description of how the *SF* distribution was derived is presented in Han et al. (2016). *IE* – represents the BaP_{eq} daily dose (μg/day); *EF* – the exposure frequency, days *per* year; *ED* – exposure duration for adults approximated to 53 years following Wu et al. (2015); *BW* – body weight (79.1 ±5.4 kg) following the US EPA (2011); *AT* – represents the average lifetime exposure for men (73.6 years = 26,864 days) and women (81.6 years = 29,784 days), the residents of Poland (GUS 2015); and *cf.* – conversion factor of 10^{-3} (mg/μg).

Since some of the parameters used in the calculations are PDFs, the quantitative assessment of cancer risk was performed by employing the Monte Carlo Simulation in Crystal Ball 7.3 software made by Decisioneering (2007). The expected risk interval was established at the 90th and 10th percentiles of the final distribution. To check for factors most affecting the output, a sensitivity analysis was performed. The diagrams of the percentage contribution of each exposure variable to the total variance of the predicted cancer risk demonstrate the findings from the sensitivity analysis. According to the US EPA (2011), one in a million chance of developing a

Table 3 Equation inputs for 1D Monte Carlo analysis

Symbol (unit)	Type	Pollution source	PM (μm)	Food loading	Probability density function or point estimate
Chemical concentration (μg/m^3)	LN	Gas grill	<2.5	Loaded	(4.60E-05;1.03E-04)[a, b]
				Unloaded	(4.08E-05;9.64E-05)[a, b]
			2.5–100	Loaded	(8.29E-05;2.17E-04)[a, b]
				Unloaded	(6.38E-05;1.64E-04)[a, b]
		Grill powered by lump charcoal	<2.5	Loaded	(3.44E-01;1.06E-00)[a, b]
				Unloaded	(4.10E-05;9.33E-05)[a, b]
			2.5–100	Loaded	(7.35E-04;1.02E-03)[a, b]
				Unloaded	(6.49E-05;1.64E-04)[a, b]
		Grill powered by charcoal briquettes	<2.5	Loaded	(4.23E-01;1.14E-00)[a, b]
				Unloaded	(5.65E-03;1.77E-02)[a, b]
			2.5–100	Loaded	(1.14E-02;3.47E-02)[a, b]
				Unloaded	(3.89E-04;5.87E-04)[a, b]
IR (m^3/h)	LN				(22.8;0.9)[a,b]
ET (hours/day)	UN				(1.0;5.0)[c,d]
EF (days/year)	UN				(20;40)[c,d]
ED (years)	PE				53
BW (kg)	LN				(79.1;5.4)[a,b]
AT (days)	PE				27,977 (days)[e]
CSF_{inh} (mg × kg/day)	LN				(3.14;1.8)[a,b]

IR inhalation rate, *ET* exposure time, *EF* exposure frequency, *ED* exposure duration, *BW* body weight, *AT* average lifetime exposure for both men and women, CSF_{inh} inhalation cancer slope factor. *LN* log-normal distribution, *UN* uniform distribution, *PE* point estimate; [a]geometric mean, [b]geometric SD, [c]minimum, and [d]maximum (GUS 2015)

human cancer over a 70-year lifetime (ILCR $= 10^{-6}$) is considered an acceptable risk, whereas a lifetime risk of one in thousand or greater (ILCR $= 10^{-3}$) constitutes a serious health threat. The results of risk analysis were presented in Table 4.

2.5 Respiratory Tract Deposition Model

During barbecues, the grill emission gases are mixed with a volume of ambient air and next this air mass is delivered into the lungs. To predict the percentage of BaP_{eq} dose deposited in the airways, the multiple-path particle dosimetry (MPPD) model (version 2.11; Applied Research Associates, Inc.; Albuquerque, NM) was employed, as described by Anjilvel and Asgharian (1995). The deposition was calculated as the sum of partial depositions in three different functional areas: head region extending from the nasal cavity to the trachea; tracheo-bronchial region, and the gas exchange pulmonary region. Deposition fractions for each bin size in different compartments of the respiratory tract are shown in Figs. 1 and 2. The calculation of PM-bound PAHs (here BaP_{eq}) distribution in the airways was done by integrating the result of size fractionated PAHs concentration (Table 1) and the deposition fractions. All calculations were performed for the hypothetical barbecue operator/consumer using the lung geometry specific for >21-year-old adult. The following breathing parameters were considered in calculations: tidal volume – 477.2 cm^3; functional residual capacity – 2792.5 cm^3; upper respiratory tract volume – 42.3 cm^3; and respiratory minute volume 6.7 dm^3/min. A polydisperse aerosol model was employed, with a particle density of 1 g/cm^3. The simulation results were provided only for regional depositions using nasal breathing pattern; calculations did not include the particle

Table 4 Estimated incremental life cancer risks (ILCR) in adults subjected to PAHs emitted from barbecue grills

Scenario	Distribution parameter	$PM_{2.5}$						$PM_{2.5–100}$					
		1	2	3	4	7	8	1	2	3	4	7	8
		G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]
		Without food			Loaded with food			Without food			Loaded with food		
Maximum exposure time	10% percentile	2.12E-05	2.13E-05	2.93E-03	2.38E-05	1.78E-01	2.19E-01	3.31E-05	3.37E-05	2.02E-04	4.3E-05	3.81E-04	5.91E-03
	90% percentile	1.71E-04	1.72E-04	2.37E-03	1.94E-02	1.44E-00	1.77E-00	2.67E-04	2.72E-04	1.63E-03	3.47E-04	3.08E-03	4.77E-02
	Geometric mean	8.38E-05	8.43E-05	1.16E-02	9.44E-05	7.01E-01	8.68E-01	1.31E-04	1.33E-04	7.99E-04	1.7E-04	1.51E-03	2.34E-02
	Geometric SD	7.51E-05	7.56E-05	1.04E-02	8.47E-05	6.34E-01	7.78E-01	1.18E-04	1.20E-04	7.16E-04	1.53E-04	1.35E-03	2.01E-02
Reduced exposure time	10% percentile	1.28E-08	1.36E-08	1.28E-06	1.45E-08	6.34E-05	8.07E-05	1.79E-08	1.49E-08	1.81E-07	2.20E-08	4.06E-07	2.05E-06
	90% percentile	2.63E-06	2.13E-06	3.22E-04	2.96E-06	1.90E-02	2.3E-02	3.56E-06	3.72E-06	2.56E-05	5.48E-06	4.44E-05	6.26E-04
	Geometric mean	1.10E-06	9.96E-07	1.3E-04	1.55E-06	8.66E-03	1.01E-02	1.62E-06	2.06E-06	1.01E-05	2.61E-06	1.96E-05	3.38E-04
	Geometric SD	3.41E-06	2.76E-06	3.27E-04	6.06E-06	3.05E-02	2.89E-02	4.71E-06	1.41E-05	2.24E-05	1.12E-05	4.89E-05	1.20E-03

[a]G – grill powered by gas
[b]LC – grill powered by lump charcoal
[c]B – grill powered by charcoal briquettes

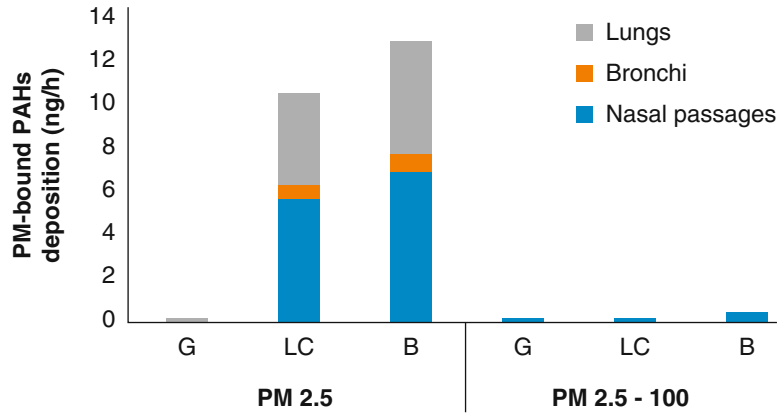

Fig. 1 Deposition of $PM_{2.5}$ and $PM_{2.5–100}$-bound polycyclic aromatic hydrocarbons (PAHs; BaP_{eq}) in the alveolar–lung region (*grey*), bronchial region (*orange*), and nasal passages (*blue*) during exposure to barbecue emission gases released from different types of barbecue installations; *G* gas grill, *LC* grill powered by lump charcoal, and *B* grill powered by charcoal briquettes in the condition with food loaded

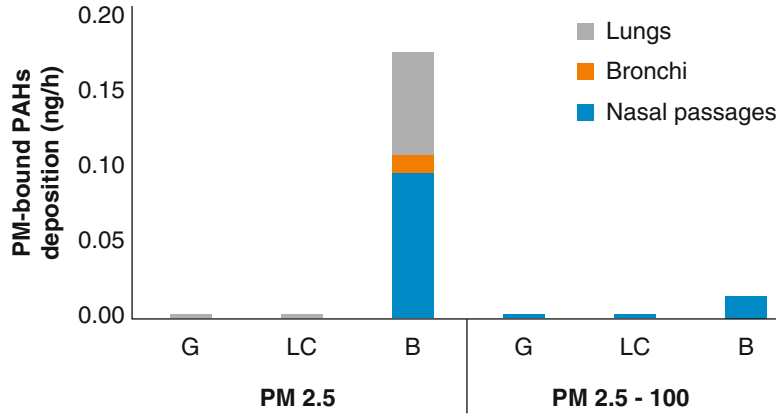

Fig. 2 Deposition of $PM_{2.5}$ and $PM_{2.5–100}$-bound polycyclic aromatic hydrocarbons (PAHs; BaP_{eq}) in the alveolar–lung region (*grey*), bronchial region (*orange*), and nasal passages (*blue*) during exposure to barbecue emission gases released from different types of barbecue installations; *G* gas grill, *LC* grill powered by lump charcoal, and *B* grill powered by charcoal briquettes in the condition without food loaded

clearance. The detailed algorithm forming the MPPD model is available in the study of Anjilvel and Asgharian (1995).

3 Results and Discussion

3.1 Particle Emissions from Barbecue Grills

PM emissions from grills are high and may locally significantly contribute to the ambient air pollutants, both fine and coarse fractions (Rogula-Kozłowska et al. 2013). The results of $PM_{2.5}$ and $PM_{2.5–100}$ concentrations released from various types of barbecue installations are summarized in Fig. 3. Each of the three barbecue installations investigated resulted in a fairly different PM release. The highest $PM_{2.5}$ concentrations were noted for the grills powered by lump charcoal and charcoal briquettes. After food loading, $PM_{2.5}$ from lump charcoal grill rose about 18 times and that from briquettes rose two times compared to the non-loading conditions. The same was observed when barbecuing with gas. Although the increase was

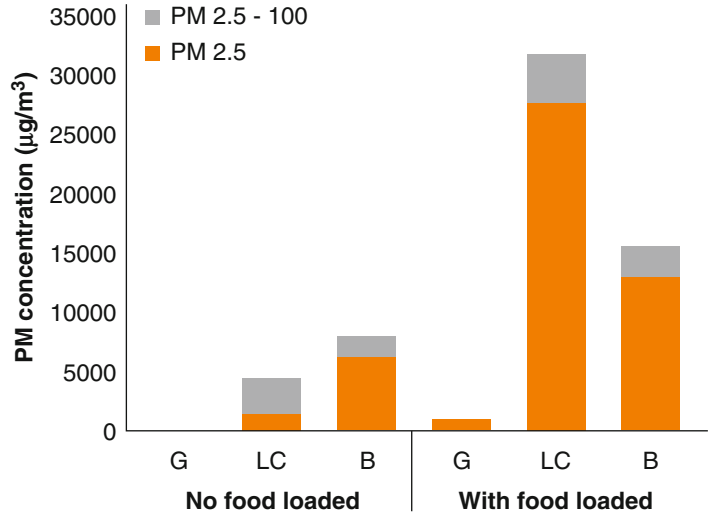

Fig. 3 $PM_{2.5}$ and $PM_{2.5–100}$ concentration in grill emission gases released from different types of barbecue installations; *G* gas grill, *LC* grill powered by lump charcoal, and *B* grill powered by charcoal briquettes, with and without food loaded

here up to 213 times, i.e., three orders of magnitude, the $PM_{2.5}$ emitted from the unloaded gas grill was only 5 µg/m^3. This observation clearly demonstrates that the use of an indirect heat source and lower temperature, typical for gas grill, significantly eliminates charring and soot formation. It was also observed that lump charcoal produced much less coarse particles than the briquettes did. Concerning the fine particles, this dependence was reversed, but only during food processing.

3.2 Particle-Bound PAHs in Barbecue Emissions

The measurements of PM-bound PAHs, showing both the average and the sum of 16 PAHs concentrations are displayed in Table 1. The comparison of the PM-bound concentration of individual PAHs for each type of grill, i.e., gas, lump charcoal, and charcoal briquette powered installations, indicates that the highest concentration of PAHs was released while using charcoal briquettes. Those concentrations ranged from 5.6 to 21.5 µg/m^3 in case of $PM_{2.5}$-bound PAHs and from 2.7 to 7.2 µg/m^3 in case of coarse $PM_{2.5–100}$-bound PAHs. Much lower emissions concerned the gas grill, with the concentration range of 0.40–0.46 µg/m^3 for $PM_{2.5}$ and 0.51–0.66 µg/m^3 for $PM_{2.5–100}$. PAHs emission from charcoal briquettes contributed to 0.16% of the total $PM_{2.5}$ mass and 0.29% of $PM_{2.5–100}$ mass in the condition with food loaded. Thus, a greater mass of the loaded PAHs concerned the coarse $PM_{2.5–100}$ fraction. Concerning the PM samples originating from the gas grill, the concentration of all kinds of PAHs was very small or below the detection limit. Similar results were obtained for the lump charcoal emission products discharged into the atmosphere, but the low PAHs concentration occurred there only when no food was thermally processed. Many studies indicate that variations in PM-bound PAHs are due mainly to the type of heat source and also the temperature and duration of cooking (Farhadian et al. 2010; FEHD 2004). In the present study, among the carcinogenic PAHs (PAH8): chrysene (5.6 µg/m^3), benz[a]anthracene (5.08 µg/m^3), and benzo[b]fluoranthene (5.58 µg/m^3) showed the highest mean values. Those agents were emitted mostly from charcoal briquettes, which may be explicable by the combustion temperature and chemical composition of briquettes. It is known that the hotter the temperature the greater is PAHs formation. Since charcoal lumps usually produce more charcoal powder, which fills up the

gaps between charring chunks and blocks the airflow, they burn at cooler temperature compared to briquettes. The oval shape of briquettes facilitates air supply making them burn at higher temperature, which enhances PAHs production. The use of gas, which does not contain PAH, and the prevailing temperature in the gas combustion chamber of approx. 200 °C were unfavorable for PM-bound PAHs formation. Thus, none of the 16 analyzed PAHs were detected in gas grill emissions. In PM samples collected from lump charcoal combustion, 12 PAHs were easily identified, including the highly carcinogenic dibenzo(a,h)anthracene. The profile of identified PAHs was much differentiated in each case, but when no food was processed the PM composition lacked PAHs of the highest carcinogenic potential. Taking into account the concentration of respirable $PM_{2.5}$ particles and the $PM_{2.5}$-bound PAHs, it can be stated that the gas grill is characterized by the lowest emissions of harmful compounds; essentially their negligible concentrations when solely gas was burning and relatively small concentrations while food processing. The gas grill creates little or no smoke due to almost complete combustion. Beside gas clean-burning, the gas chamber construction facilitates the maintaining of constant combustion temperature, which helps protect food from charring and thus PAHs formation.

The PAHs concentrations emitted from barbecue grills observed in the present study were much higher than the mean values of these pollutants reported in the Upper Silesia ambient air (Kozielska and Rogula-Kozłowska 2014), but much lower compared to the indoor restaurant values reported by Li et al. (2003). Between 1992 and 2005, the sum of 9 PM-bound PAHs (Fl, BaA, Ch, BbF, BkF, BaP, DBA, BghiP, IP) in the city of Katowice in southern Poland city was 0.153 μg/m^3, while the average concentration of 16 PAHs measured in the above-mentioned study by Li et al. (2003) in Chinese, Japanese, Western-style, and fast food restaurants was 68.41 μg/m^3. Additionally, charcoal briquettes burn for a longer period of time than gas does, emitting more CO_2 into the atmosphere. When comparing the carbon footprints of two major grill types, Johnson (2009) has found that CO_2 emission during grilling using charcoal is almost three times as large as that during grilling using liquid propane gas. Compared with charcoal, gas has a low carbon-to-hydrogen ratio and a low amount of tar, which means that it generates smaller amounts of carbon dioxide and other carbon-based pollutants *per* unit of heat produced. A comparison made by Rose et al. (2015) has determined the PAHs formation in barbecue foods cooked over different fuel types (charcoal, charcoal plus wood chips, briquettes, and gas). The authors demonstrate that charcoal- and woodchip-fueled barbecues have shown the highest concentration of PAHs, while gas and briquettes barbecues the smallest, which is somewhat at variance with the present results concerning the briquettes. The discrepancy may be in part attributed to the way in which briquettes and lump release ash residues into the air during combustion. Briquettes produce more charred remains and ashes that possibly cover food, at the same time 'protecting' food matrix from absorbing gaseous PAHs. In case of non-dusty lump charcoal, food remains uncovered by ash, which fosters PAHs penetration into food matrix.

3.3 Risk Assessment

Daily inhalation exposure to all 16 PAHs was calculated according to Eq. 2. Table 5 shows the results of these calculations presented as geometric means. The difference in PAHs concentration among charcoal, gas, and briquettes was significant, ranging from 0.04 to 401.6 ng/day. The greatest difference was noted when examining the exposure in terms of the particle size. The exposure to $PM_{2.5}$-bound PAHs was much greater compared to $PM_{2.5–100}$-bound PAHs and was the greatest when the food was grilled over the briquettes. Similar inhalation exposure rates were evaluated in a study of Zhang et al. (2015) who measured 16 PAHs among consumers of night markets in China, where barbecue grilling was the primary cooking method. Those authors found that the BaP-adjusted PAHs was

Table 5 Daily inhalation intake of PAHs (ng/day) resulting from exposure to barbecue emissions

Distribution parameter	$PM_{2.5}$						$PM_{2.5–100}$					
	1	2	3	4	7	8	1	2	3	4	7	8
	G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]	G[a]	LC[b]	B[c]
	Without food			Loaded with food			Without food			Loaded with food		
Geometric Mean	0.04	0.04	5.37	0.04	326.9	401.6	0.06	0.06	0.37	0.08	0.70	10.83

[a]G – grill powered by gas
[b]LC – grill powered by lump charcoal
[c]B – grill powered by charcoal briquettes

1750 ± 878 ng/day, with a range of 451–3430 ng/day, indicating a high potential risk. Discrepancies between the results of the present study and those reported by Zhang et al. (2015) were due rather to different assumptions concerning the exposure duration than to different PAHs concentrations. It is hard to state clearly which of the exposure routes – oral, dermal, or inhalation – have the greatest contribution in shaping PAHs migration from barbecues into the human body. Other studies demonstrate that inhalation exposure to PAHs from barbecues predominates over the other routes. For instance, Alomirah et al. (2011) have estimated that the total mean dietary intake of 16 most important PAHs, according to US EPA's (2011) data, is 1108 ng/day, which is twice as much as the maximum inhalation intake found in the present study. Nonetheless, a widely discussed problem of air pollutants' influence on morbidity and mortality risk may have the additional aspect barbecue-related pollution. Taking into account that nearly 50,000 people die prematurely in Poland every year due to the exposure to $PM_{2.5}$ and there are over 1100 premature deaths a year in the biggest 11 cities in Poland due to lung cancer attributable to the exposure to $PM_{2.5}$ (Badyda et al. 2016), the widespread use of traditional barbecues may have its contribution to this state of affairs.

Looking into the percentile predictions of the incremental lifetime cancer risk determined from the cumulative density functions it is evident that the shape of risk distribution was log-normal in all tested groups. Since the risk scenario is fairly complicated, we chose to present the probability density functions in the form of geometric means ± geometric standard deviations (Table 4) rather than in a graphic diagram. Depending on PM bounding, food loading condition, and the type of fuel used for grill powering, the total the incremental lifetime cancer risk ranged between the 10^0 and 10^{-5} level. The findings obtained, even a four-order of magnitude higher risk in case of exposure to briquette emission products compared to gas exhausts, suggest that complete combustion plays a significant role in protecting barbecue consumers against inhalation exposure. It also was observed that as the size distribution shifts toward smaller particles, the risk increases. The incremental lifetime cancer risk values were the greatest in case of exposure to $PM_{2.5}$-bound PAHs released from charcoal briquettes (log-normal distribution; $8.68 \times 10^{-1} \pm 7.78 \times 10^{-1}$), while the smallest risks concerned exposure to gas incineration products ($8.38 \times 10^{-5} \pm 7.51 \times 10^{-5}$). A relative contribution of each fuel type to the PAHs-induced inhalation risk was in the following ascending order: gas, lump charcoal, and charcoal briquettes. In case of briquettes and lump charcoal, the risk exceeded the permissible 10^{-3} limit of acceptability, which should prompt the taking of preventive measures. It should be noted that the present discussion is under the assumption of maximum grill operator exposure. A real exposure is in fact a fraction of the calculated exposure, as it concerns the operator being in front of the grill. To check whether a shortening of exposure time only to one hour and one barbecue

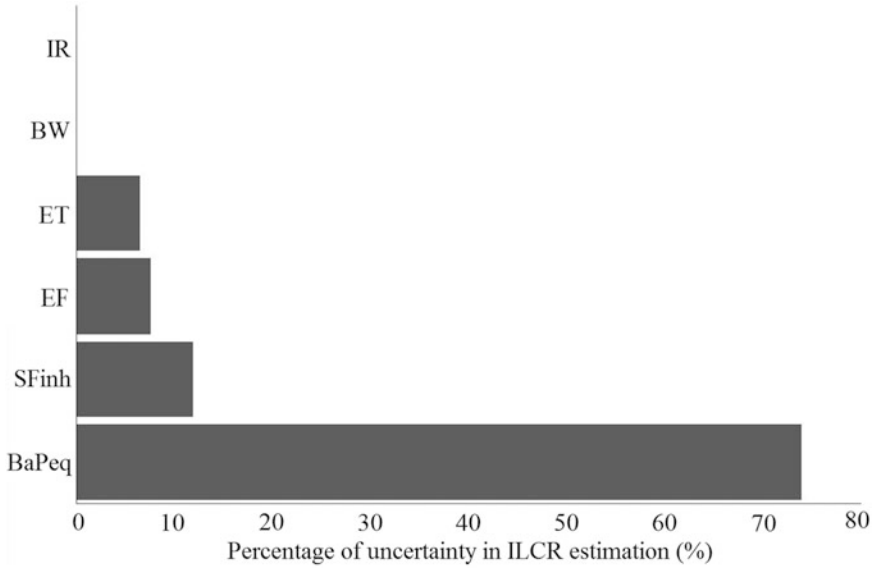

Fig. 4 Uncertainty in the estimation of the incremental lifetime cancer risk (ILCR) brought about by the interference of various parameters; *IR* inhalation rate (m^3/day), *BW* body weight (kg), *ET* exposure time (hours/day), *EF* exposure frequency (days/year), *CSFinh* inhalation cancer slope factor (kg·day/mg), BaP_{eq} benzo[a]pyrene carcinogenic equivalent (mg/m^3)

meeting *per* year would cause a reduction in risk, some additional simulations were performed. These results are presented in Table 4. It is concluded that exposure time reduction does not significantly influence the risk results. Although in case of exposure to gas and lump charcoal emissions, the incremental lifetime cancer risk would be lower by about one order of magnitude, the risk induced by briquettes, with food loaded grill, still remains above the acceptable 10^{-3} limit level. The influence of exposure time to the final risk estimation is much smaller compared to the sole effect of PAHs concentration. A confirmation of this conclusion is presented in the form of a sensitivity graph (Fig. 4), indicating that the greatest percentage uncertainty, amounting to 74%, in the estimation of the incremental lifetime cancer risk is brought about by the PAHs (BaP_{eq}) concentration; even concerning the scenario with the lowest PAHs concentrations; i.e., gas grill.

3.4 Deposition of PM-Bound PAHs in the Respiratory Tract

PM-bound PAHs penetrate into the respiratory system with different efficacy, depending on the size of its PM carriers. Thus, in the present study the size-dependent deposition was designated independently for fine and coarse particles. The total PAHs deposition was more than ten times greater when grills were loaded with food. Figures 1 and 2 show the relative carcinogenic concentrations of BaP_{eq} in the respiratory tract. Small amounts of PAHs were deposited in case of exposure to $PM_{2.5–100}$ particles. That is explicable by the fact that the semi-volatile organic pollutants, with a low vapor pressure, like PAHs, are generally associated with ultrafine particles (Offenberg and Baker 1999), which also is in line with the data presented in Table 3. The highest total deposition occurred while grilling food using briquettes (13 ng/h) and lump charcoal

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(10.5 ng/h). About half of the $PM_{2.5}$-bound PAHs was small enough to pass through the respiration system and being deposited in the lungs. The alveolar region is particularly vulnerable to PM-bound pollutants since it lacks the mucociliary clearance which facilitates further particle translocation (Löndahl et al. 2014).

4 Conclusions

The study demonstrates that barbecue emissions are a substantial source of atmospheric pollution by PAHs. The type of heat source used for grilling influences the PM-bound PAHs formation. The highest concentration of $PM_{2.5}$-bound PAHs has been found while burning charcoal briquettes. Nonetheless, it seems uncertain whether it would be preferable to use lump charcoal instead of briquettes for powering of traditional grills. Although a higher amount of $PM_{2.5}$-bound PAHs was emitted when burning briquettes, lump charcoal produces a much greater concentration of $PM_{2.5}$ particles, possibly loaded with other than PAHs toxic compounds. Loading a grill with food generates much more PM-bound PAHs emissions. While comparing the exposure to PM and PM-bound PAHs from the grill sources to other domestic and non-domestic sources, like home activities, cigarette smoking, or commuting to work, the grill exposure is fairly substantial. The findings highlight that traditional grilling poses lung cancer risk above the acceptable limits. An effective preventive strategy against emissions while burning charcoal and briquettes, especially in a poorly ventilated space, should be implemented. Labeling briquette bags with the information on the potential carcinogenicity or installing a simple emission control device in barbecuing facilities, could reduce the health risk from barbecue emissions.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 29–36
DOI 10.1007/5584_2017_50
© Springer International Publishing Switzerland 2017
Published online: 2 June 2017

Intensity of Anxiety and Depression in Patients with Lung Cancer in Relation to Quality of Life

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Abstract

Psychological factors, such as the anxiety and depression, which often occur in patients with lung cancer might negatively influence their quality of life. The aim of the study was to evaluate the effect of anxiety and depression in lung cancer patients on quality of life. The study included 180 lung patients of the mean age of 62.7 ± 9.7 years. The following scales were employed in the study: Quality of Life Questionnaire QLQ-C30 and LC13 scale, and Hospital Anxiety and Depression scale (HADS). The overall score of quality of life measured by QLQ-C30 was 47.1 ± 23.4 points on a hundred-point scale. Anxiety was diagnosed in 67 patients (37.2%) and depression in 75 patients (41.7%) by HADS. Quality of life was significantly worse in case of anxiety and depression ($p < 0.05$), which negatively influenced both functional and symptom intensity scales measured with QLQ-C30 and QLQ-LC13. We conclude that early identification of anxiety and depression may help in therapeutic decision-making and may be a useful predictive factor in lung cancer patients.

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Keywords
Anxiety • Depression • Lung cancer • Psychological factors • Quality of life • Questionnaire survey

1 Introduction

Lung cancer is typically diagnosed late and is associated with unfavorable prognosis. In most cases, lung cancer is diagnosed when it is already advanced, and many patients require palliative care (Pula et al. 2014). Due to delayed diagnosis, 5-year survival rate amounts to only 15%. The treatment includes surgery, adjuvant chemotherapy, and radiation therapy; however, it is burdened with many side effects. Therefore, disease progression and the severity of symptoms and side effects decrease the quality of life (QoL) in this group of patients (Liao et al. 2014; Iyer et al. 2013; Zimmermann et al. 2011; Mor et al. 1994).

In recent decades, an increasing amount of attention has been devoted to the holistic approach to cancer, which includes the evaluation of not only symptom intensity, survival, and satisfaction from treatment, but also the QoL in this group of patients. The focus has also been given to psychological disorders. When cancer is diagnosed and treatment begins physical capacity decreases, and social and family interactions become limited; the patient develops a sense of loss and mental discomfort, usually in the form of depression or anxiety (Trzebiatowska 2000). Cancer strongly affects all spheres of life, and the consequences of the disease are not only biological but also psychological and social.

Anxiety may be present before the diagnosis of cancer, when the first worrying symptoms occur, and it remains with the patient throughout the treatment. The level of depression and anxiety can be seen as a QoL predictor. Some studies report that one in four individuals experiences depression or other psychological problems during their cancer treatment (Carlsen et al. 2005). Depression affects an average of 23–40% of patients with lung cancer, while fear and anxiety are diagnosed in 16–23% of this group (Hopwood and Stephens 2000). Anxiety and depression are related to QoL of lung cancer patients. The level of anxiety often increases during chemotherapy (Li et al. 2012). Depression is also a predictor of shorter survival in patients with recently diagnosed lung cancer (Pirl et al. 2012). Therefore, in this study we seek to determine the intensity of anxiety and depression in lung cancer patients and determine their effect on QoL in this group of patients.

2 Methods

2.1 Patients

This is a survey-type study that included 180 patients aged 62.7 ± 9.7 (F/M – 82/98), hospitalized due to lung cancer in the Lower Silesian Center for Lung Diseases in Wroclaw, Poland. The mean number of patient hospitalizations was 1.0 ± 1.7. The study was approved by a local Bioethics Committee (permit no. 507/2015). All patients gave written informed consent to complete the questionnaires and were informed that they could withdraw from the study at any stage.

The following inclusion criteria were used:

- lung cancer with histopathological confirmation;
- age > 18 years;
- consent to participate;
- understanding the questions included in the questionnaire.

The exclusion criteria were as follows:

- uncertain cancer diagnosis;
- coexistence of other severe chronic diseases that could influence the patient's perception of health status such as other malignant tumors,

heart failure, chronic obstructive pulmonary disease, asthma, and hemodynamic instability;
- cognitive impairment indicative of dementia.

The patients' socio-demographic and clinical data were gathered from the medical records available. Data on the time of lung cancer diagnosis, treatment administered, tumor type, TNM classification, spirometry results, genetic predisposition to cancer, tobacco smoking, and other essential socio-demographic information were directly obtained from the patients.

2.2 Survey Tools

We used the quality of life questionnaire (QLQ-C30) and its QLQ-LC13 (version 3.0) module of the European Organization for Research and Treatment of Cancer (EORTC) for the evaluation of lung cancer patients (Bergman et al. 1994). The questionnaire is designed to evaluate the patients' self-assessed health status and social, physical, and emotional functioning. It consists of 30 questions grouped into five functional scales: physical functioning (5 items), role functioning (2 items), emotional functioning (4 items), cognitive functioning (2 items), and social functioning (2 items). It also includes three symptom scales: fatigue (3 items), nausea and vomiting (2 items), and pain (2 items), and six single questions evaluating the severity of the following symptoms: dyspnea, insomnia, appetite loss, constipation, diarrhea, and financial difficulties. The last two questions pertain to the global health status assessment. Respondents provide answers using the following four-grade scale: never – 1, sometimes – 2, often – 3, and very often – 4. The QLQ-LC13 module contains 13 items concerning typical lung cancer symptoms.

Performance status was analyzed using the Zubrod scale, which runs from 0 to 5, where 0 denotes lack of disease symptoms, 1 – symptomatic, reduced activity, patient able to carry out light work, 2 – symptomatic, patient capable of all self-care, unable to carry out any work activities, resting for as much as 50% of the day, 3 – patient capable of only limited self-care, resting in bed for more than 50% of the day, 4 – patient confined to bed at all times, incapable of any self-care, and 5 – death.

We also used the hospital anxiety and depression scale (HADS) that evaluates anxiety and depression without investigating somatic symptoms. This scale consists of 14 items, seven of which are related to anxiety (HAD-A), while another seven are related to depression (HAD-D). A score < 8 points indicates the lack of any mental disorder, ≥ 8 indicates that a disorder is probably present, and > 10 indicates that a disorder is highly likely to be present (Bjelland et al. 2002; Zigmond and Snaith 1983).

2.3 Statistical Elaboration

Data were presented as means $\pm$SD. Distribution of normality was checked with the Shapiro-Wilk test. Differences in quantitative variables between two groups were evaluated Student's *t*-test or Mann-Whitney U test as appropriate. In case of more than two groups, one-way ANOVA or Kruskal-Wallis test was used as appropriate. When significant differences were unrevealed a *post-hoc* analysis Tukey's HSD test (normal distribution) or Bonferroni-corrected Mann-Whitney U test (skewed distribution) were employed.

Correlations between two quantitative variables were analyzed using Pearson's coefficient (when both variables had normal distribution) or Spearman's coefficient (when the distribution of at least one variable was skewed).

The strength of dependence was interpreted according to the following scheme:

- $r \geq 0.9$ – very strong dependence,
- $0.7 \leq r < 0.9$ – strong dependence,
- $0.5 \leq r < 0.7$ – moderate dependence,
- $0.3 \leq r < 0.5$ – weak dependence,
- $r < 0.3$ – very weak (negligible) dependence.

A p-value <0.05 was used to define statistically significant differences. The analysis was conducted using R software, version 3.3.1.

3 Results

3.1 Baseline and Demographic Data

Spirometry revealed the following results: mean forced expiratory volume in 1 s (FEV1) was 2.38 ± 0.8 L, mean forced vital capacity (FVC) amounted to 3.06 ± 0.96 L, and the Tiffeneau-Pinelli index (FEV1/FVC) was $79.5 \pm 20.4\%$. As for the socio-demographic factors, 105 patients (58.3%) were in relationship, while 75 (41.7%) were single. Nearly half of the patients had vocational education (88 patients; 48.9%), 59 patients (32.8%) had secondary education, 18 patients (10.0%) had higher education, and 15 patients (8.3%) had primary education. The patients' occupational status was as follows: 121 patients (67.2%) received unemployment benefits and disability or retirement pension; 53 patients (29.4%) had a permanent job, and 6 patients (3.3%) lived on family support. Concerning comorbidities, 81 patients (45.0%) had one concomitant disease, 38 (21.1%) had two concomitant diseases, two (1.1%) had three, and 59 patients (32.8%) had no comorbidities. Forty three patients (23.9%) had metastasis to one organ, while 20 patients (11.1%) had multiple organ metastases. No metastasis was found in 117 respondents (65.0%). In 72 patients (40.0%), only surgical treatment was performed, while the remaining 60.0% of patients 108 were treated with other methods. On the Zubrod scale of performance status, 33 patients (18.3%) scored 0, 79 patients (43.9%) scored 1, 58 (32.2%) patients scored 2, nine patients (5.0%) scored 3, and four patients (0.6%) scored 4. The data collected from 180 patients are presented in Table 1.

Table 2 shows the incidence of anxiety and depression disorders in cancer patients. Anxiety, either pronounced or borderline, was present in 113 patients and depression in 105 patients out of the 180 patients investigated, which is a comparable incidence.

3.2 Anxiety and Quality of Life

There were significant changes in 24 out of the 26 QoL scales, pointing to the anxiety as an influential factor shaping the life quality. A *post-hoc* analysis gave an insight into the nature of this dependence, revealing that patients without psychological disorders fared significantly better than those with borderline and full-fledged disorders in the following way (Table 3):

- had a better general QoL;
- had a better role functioning;
- had a better physical, emotional, cognitive, and social functioning; patients with borderline disorders functioned better than those with full-fledged disorders;
- were less affected by fatigue, pain, and financial difficulties; patients with borderline disorders had significantly better results than those with full-fledged disorders;
- were less affected by constipation and chest pain;
- were less affected by body pain;
- were less affected by nausea and vomiting, dyspnea, insomnia, appetite loss, diarrhea, cough, mouth or tongue soreness, dysphagia, peripheral neuropathy, alopecia, and arm or shoulder pain.

3.3 Depression and Quality of Life

There were significant changes in 23 out of the 26 QoL scales, pointing to depression as another factor shaping the life quality. A *post-hoc* analysis revealed that patients without psychological disorders fared significantly better than those with borderline and full-fledged disorders in the following way (Table 4):

– had a better general QoL; patients with borderline disorders had better general QoL than those with full-fledged disorders;

Table 1 Characteristics of cancer patients

Variable	Mean ± SD	Median (quartiles)
Age (years)	62.7 ± 9.7	63.0 (58.0–68.0)
No. of hospitalizations	1.0 ± 1.7	0 (0–1)
FEV1 (L)	2.38 ± 0.80	2.20 (1.84–2.84)
FVC (L)	3.06 ± 0.96	2.92 (2.31–3.65)
FEV1/FVC (%)	79.5 ± 20.4	77.2 (69.2–85.6)
Gender	**n (%)**	
Women	82 (45.6)	
Men	98 (54.4)	
Marital status:		
In relationship	105 (58.3)	
Single	75 (41.7)	
Education		
Primary	15 (8.3)	
Vocational	88 (48.9)	
Secondary	59 (32.8)	
Higher	18 (10.0)	
Occupational status		
Permanent job	53 (29.4)	
Unemployment, pension	121 (67.2)	
Family support	6 (3.3)	
Comorbidities		
None	59 (32.8)	
1 disease	81 (45.0)	
2 diseases	38 (21.1)	
3 diseases	2 (1.1)	
Metastases		
None	117 (65.0)	
One organ	43 (23.9)	
Multiple organs	20 (11.1)	
Treatment		
Surgical only	72 (40.0)	
Other	108 (60.0)	
Performance score		
0	33 (18.3)	
1	79 (43.9)	
2	58 (32.2)	
3	9 (5.0)	
4	1 (0.6)	

Table 2 Hospital anxiety and depression scale (HADS) results in cancer patients

	n (%)
A – Anxiety	
Normal	66 (36.7)
Borderline abnormal	47 (26.1)
Abnormal	67 (37.2)
B – Depression	
Normal	65 (36.1)
Borderline abnormal	40 (22.2)
Abnormal	75 (41.7)

– had better physical and role functioning, and cognitive and social functioning;
– had better emotional functioning; patients with borderline disorders functioned better than those with full-fledged disorders;
– were less affected by fatigue and pain; patients with borderline disorders had less fatigue than those with full-fledged disorders;
– were less affected by mouth or tongue soreness, peripheral neuropathy, arm or shoulder pain, and pain in other sites;
– were less affected by vomiting, dyspnea, insomnia, appetite loss, diarrhea, financial difficulties, cough, dysphagia, and chest pain;
– were significantly less affected by alopecia.

Table 3 Relationship between anxiety and quality of life (QoL) in cancer patients

Scale		Normal Median (quartiles)	Borderline Median (quartiles)	Abnormal Median (quartiles)	[a]p <
QLQ-C30 functional scales	Global QoL	66.7 (50.0–83.3)	41.7 (25.0–54.2)	33.3 (25.0–41.7)	0.001
	Physical functioning	86.7 (75.0–93.3)	66.7 (60.0–86.7)	66.7 (60.0–73.3)	0.001
	Role functioning	100.0 (66.7–100.0)	66.7 (50.0–66.7)	50.0 (33.3–66.7)	0.001
	Emotional functioning	91.7 (75.0–100.0)	41.7 (33.3–70.8)	33.3 (25.0–50.0)	0.001
	Cognitive functioning	100.0 (83.3–100.0)	83.3 (66.7–83.3)	66.7 (50.0–83.3)	0.001
	Social functioning	100.0 (83.3–100.0)	66.7 (33.3–83.3)	33.3 (33.3–66.7)	0.001
QLQ-C30 symptom scales	Fatigue	22.2 (11.1–33.3)	44.4 (33.3–55.6)	55.6 (44.4–66.7)	0.001
	Nausea, vomiting	0 (0–0)	16.7 (0–16.7)	16.7 (0–25.0)	0.001
	Pain	16.7 (0–33.3)	33.3 (33.3–50.0)	50.0 (33.3–66.7)	0.001
	Dyspnea	33.3 (33.3–33.3)	33.3 (33.3–66.7)	33.3 (33.3–66.7)	0.001
	Insomnia	0 (0–33.3)	66.7 (33.3–66.7)	66.7 (33.3–66.7)	0.001
	Appetite loss	0 (0–33.3)	33.3 (33.3–66.7)	33.3 (33.3–66.7)	0.001
	Constipation	0 (0–33.3)	0 (0–33.3)	33.3 (0–33.3)	0.028
	Diarrhea	0 (0–0)	0 (0–16.7)	0 (0–16.7)	0.002
	Financial difficulties	0 (0–25.0)	33.3 (0–66.7)	66.7 (33.3–66.7)	0.001
QLQ-LC13	Dyspnea	11.1 (11.1–22.2)	33.3 (27.8–55.6)	33.3 (33.3–66.7)	0.001
	Cough	33.3 (33.3–66.7)	66.7 (33.3–66.7)	66.7 (33.3–66.7)	0.002
	Hemoptysis	0 (0–33.3)	0 (0–33.3)	0 (0–33.3)	0.034
	Sore mouth or tongue	0 (0–0)	0 (0–0)	0 (0–33.3)	0.001
	Dysphagia	0 (0–0)	0 (0–33.3)	33.3 (0–33.3)	0.001
	Peripheral neuropathy	0 (0–0)	0 (0–33.3)	0 (0–33.3)	0.001
	Alopecia	0 (0–0)	0 (0–33.3)	0 (0–33.3)	0.003
	Pain in chest	0 (0–33.3)	33.3 (0–33.3)	33.3 (16.7–50.0)	0.001
	Pain in arm or shoulder	0 (0–0)	0 (0–33.3)	0 (0–33.3)	0.001
	Pain in other parts	0 (0–33.3)	33.3 (0–33.3)	33.3 (0–66.7)	0.001
	Pain medication	75.0 (0–93.8)	50.0 (0–75.0)	75.0 (50.0–75.0)	0.650

[a]Kruskal-Wallis test

Table 4 Relationship between depression and quality of life (QoL) in lung cancer patients

Scale		Normal Median (quartiles)	Borderline Median (quartiles)	Abnormal Median (quartiles)	[a]p <
QLQ-C30 functional scales	Global QoL	66.7 (50.0–83.3)	41.7 (33.3–50.0)	33.3 (25.0–41.7)	0.001
	Physical functioning	86.7 (80.0–93.3)	66.7 (53.3–80.0)	66.7 (60.0–73.3)	0.001
	Role functioning	100.0 (66.7–100.0)	66.7 (50.0–66.7)	50.0 (33.3–66.7)	0.001
	Emotional functioning	91.7 (66.7–100.0)	50.0 (33.3–75.0)	33.3 (25.0–50.0)	0.001
	Cognitive functioning	100.0 (83.3–100.0)	66.7 (50.0–83.3)	66.7 (50.0–83.3)	0.001
	Social functioning	100.0 (83.3–100.0)	66.7 (33.3–83.3)	33.3 (33.3–66.7)	0.001
QLQ-C30 symptom scales	Fatigue	22.2 (11.1–33.3)	44.4 (33.3–55.6)	55.6 (44.4–66.0)	0.001
	Nausea and vomiting	0 (0–0)	0 (0–16.7)	16.7 (0–33.3)	0.001
	Pain	16.7 (0–33.3)	33.3 (33.3–50.0)	50.0 (33.3–66.7)	0.001
	Dyspnea	33.3 (33.3–33.3)	33.3 (33.3–66.7)	66.7 (33.3–66.7)	0.001
	Insomnia	0 (0–33.3)	66.7 (33.3–66.7)	66.7 (33.3–66.7)	0.001
	Appetite loss	0 (0–33.3)	33.3 (25.0–41.7)	33.3 (33.3–66.7)	0.001
	Constipation	0 (0–33.3)	0 (0–33.3)	0 (0–33.3)	0.062
	Diarrhea	0 (0–0)	0 (0–33.3)	0 (0–0)	0.001
	Financial difficulties	0 (0–33.3)	33.3 (0–66.7)	33.3 (33.3–66.7)	0.001
QLQ-LC13	Dyspnea	11.1 (11.1–22.2)	33.3 (33.3–66.7)	33.3 (33.3–55.6)	0.001
	Cough	33.3 (33.3–33.3)	66.7 (33.3–66.7)	66.7 (33.3–66.7)	0.001
	Hemoptysis	0 (0–33.3)	0 (0–33.3)	0 (0–33.3)	0.553
	Sore mouth or tongue	0 (0–0)	0 (0–8.3)	0 (0–33.3)	0.013
	Dysphagia	0 (0–0)	0 (0–33. 3)	33.3 (0–33.3)	0.001
	Peripheral neuropathy	0 (0–0)	0 (0–33.3)	0 (0–33.3)	0.001
	Alopecia	0 (0–0)	0 (0–33.3)	0 (0–33.3)	0.003
	Pain in chest	0 (0–33.3)	33.3 (0–33.3)	33.3 (0–33.3)	0.001
	Arm & shoulder pain	0 (0–0)	0 (0–33.3)	0 (0–33.3)	0.036
	Pain in other sites	0 (0–33.3)	16.7 (0–33.3)	33.3 (0–66.7)	0.001
	Pain medication	75.0 (0–75.0)	75.0 (0–75.0)	50.0 (50.0–75.0)	0.973

[a]Kruskal-Wallis

4 Discussion

The findings of the present study add to the evidence of a relation between anxiety or depression and QoL in lung cancer patients. Although the prognostic outlook for lung cancer patients has improved in recent years, the disease still has one of the poorest outcome of all human malignancies. The knowledge that the disease is mostly incurable takes a toll on the patient after diagnosis, including increased anxiety and depression, with an associated decline in QoL (Polanski et al. 2016). Zabora et al. (2001) have reported that the prevalence of psychological distress varies by the cancer type; lung cancer has the greatest prevalence of distress amounting to 43.4%. Similar results were obtained in the present study, where the prevalence of depression in cancer patients amounted to 41.7% and that of anxiety to 37.2%. Carlsen et al. (2005) have underscored that lung cancer patients more often experience psychosocial problems during and after treatment. The results of the present study demonstrate an inverse association between the presence of anxiety or depression and QoL in lung cancer patients. An inverse association has also been observed by Arrieta et al. (2013). Those authors emphasize that depression and anxiety are associated with decreased health-related QoL scores, and depression also is independently associated with treatment adherence and poor prognosis. The present study demonstrates that patients without symptoms of depression or anxiety reported a better QoL, had fewer problems with role functioning, and displayed better results regarding

physical, cognitive, and emotional functioning. Moreover, patients free from symptoms of depression or anxiety experienced less fatigue, pain, and fewer somatic symptoms. We believe that further research in larger samples of patients is needed to confirm these findings and to gain a better insight into the relationship between anxiety, depression, and QoL.

We conclude that anxiety and depression inversely affect QoL in lung cancer patients, as demonstrated by the results in both functional and symptom scales of QLQ-C30 and QLQ-LC13 questionnaires. Early identification of anxiety and depression may influence therapeutic decision-making and may become a predictive factor in the treatment process. Therefore, we believe that psychological assessment in lung cancer patients is of essential clinical importance.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 37–46
DOI 10.1007/5584_2017_73
© Springer International Publishing AG 2017
Published online: 6 July 2017

Oleic Derivatives of Dopamine and Respiration

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Abstract

Ventilatory inhibition is considered an undesirable pharmacological side effect of pharmacotherapy in neurodegenerative conditions underlain by brain dopamine deficiency. In this context, oleic derivatives of dopamine or N-acyl-dopamines are novel substances that may be of high therapeutic interest as having the ability to cross the blood-brain barrier and acting in dopamine-like manner. In the present study we seek to define the influence of N-acyl-dopamines on lung ventilation and its hypoxic responses in the rat. We found that N-oleoyl-dopamine decreased both normoxic and peak hypoxic ventilation in response to 8% acute hypoxia, on average, by 31% and 41%, respectively. Its metabolite, 3′-O-methyl-N-oleoyl-dopamine, caused a 15% ventilatory decrease each, whereas an oleic ester derivative, 3′-O-oleoyl-N-oleoyl-dopamine, caused 11% and 19% ventilatory decreases, respectively. All three N-acyl-dopamines investigated displayed an inhibitory effect on ventilation. The findings indicate that 3′-O-methyl-N-oleoyl-dopamine and 3′-O-oleoyl-N-oleoyl-dopamine performed better than N-oleoyl-dopamine in term of less ventilatory suppression, albeit the differences among the three compounds were modest. We conclude that N-acyl-dopamines are worthy of intensified explorations as potential carriers of dopamine molecule in view of the lack of clinically effective methods of dopamine delivery into the brain in neurodegenerative conditions.

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Keywords
Brain • Dopamine • Lung ventilation • N-acyl-dopamines • Oleic derivatives of dopamine • Hypoxia • Neurodegenerative disorders

1 Introduction

Neurodegenerative disorders, related to central dopamine insufficiency, like Parkinson's disease (PD) (Cacabelos 2017), are often accompanied by ventilatory impairment (Baille et al. 2016). The issue is not only limited to the weakness of respiratory muscles (Wang et al. 2014) but also to the impairment of ventilatory chemosensitivity (Bialkowska et al. 2016; Onodera et al. 2000; Serebrovskaya et al. 1998). Dopamine is one of the most important neurotransmitters involved in the central and peripheral hypoxic chemosensitivity (Bialkowska et al. 2015). It has an inhibitory effect on carotid body-driven hypoxic ventilatory response (HVR) (Monteiro et al. 2009; Ward and Bellville 1982; Welsh et al. 1978). In contrast, there is a consistent impression that central dopamine exerts an enhancing effect on ventilation and its acute hypoxic augmentation. The neurotransmitter and its D2 receptors are present in the solitary tract nucleus that integrates the neural input relayed from the carotid chemoreceptors (Kline et al. 2002; Lawrence et al. 1995). Neuronal dopamine is released by hypoxia (Goiny et al. 1991) and central antagonism of D2 receptors suppresses hypoxic ventilation (Osanai et al. 1997; Hsiao et al. 1989). Bialkowska et al. (2016) have found that the insufficient central stimulatory DA element predominates in the mechanism of ventilatory impairment in the reserpine-induced parkinsonism in the rat.

Therapy of PD-like states remains problematic as there are a number of unresolved issues. Dopamine proper administered systemically has a fleeting peripheral action, due to rapid degradation; it does not penetrate through the blood-brain barrier, due to hydrophilicity; and the clinically practical ways of dopamine delivery into the brain are still unavailable. Further, surrogate brain dopamine-enhancing drugs currently used, such as the gold standard L-DOPA (Tambasco et al. 2011), do not decisively improve ventilation in the clinical setting and may lead to further respiratory problems with therapy time, even if other dopamine deficiency-related symptoms are reduced (Chen and Swope 2007). As ventilatory impairment jeopardizes brain and other tissues' oxygenation, which may worsen the PD-like state, novel drug candidates devoid of respiratory side effects are sought for.

One class of such drugs are lipid derivatives of dopamine, products of condensation of dopamine and fatty acids (Pokorski and Matysiak 1998). N-oleoyl-dopamine (OLDA) (Fig. 1a) is the exemplary of the class (Chu et al. 2003). It is a bioactive compound, native to the mammal brain, although with unsettled physiological role, having dopamine-like action consisting of increasing locomotion and muscle relaxation (Konieczny et al. 2009; Przegalinski et al. 2006), and decreasing ventilation (Zajac and Pokorski 2008). OLDA's synthesis in the brain resemble that of dopamine (Chu et al. 2003). OLDA enters the metabolic pathway of dopamine undergoing O-methylation *via* catechol-O-methyltransferase (COMT), yielding 3′-O-methyl-N-oleoyl-dopamine derivative (OMe-OLDA) (Fig. 1b) that maintains the bioactive properties (Zajac et al. 2014; Rekawek and Pokorski 2011). Importantly, the compounds being lipophilic, penetrate through the blood-brain barrier and thus could carry the dopamine molecule over to the brain (Zajac et al. 2006, 2012; Pokorski et al. 2003).

In the context of impaired ventilation in PD, potentially capable of aggravating the chronic tissue hypoxia-inflammatory sequelae, we deemed it warranted to define the influence on lung ventilation and its hypoxic responses of the N-acyl-dopamine compounds above outlined to

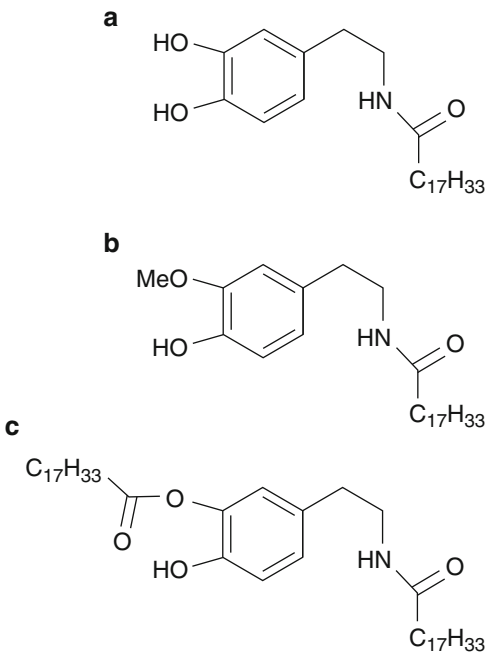

Fig. 1 Chemical structures of N-oleoyl-dopamine, OLDA (**a**), 3′-O-methyl-N-oleoyl-dopamine, OMe-OLDA (**b**), and 3′-O-oleoyl-N-oleoyl-dopamine, OL-OLDA (**c**)

choose the optimal derivative, in terms of the least ventilation hampering action, for further elaboration on the biological action of this class of compounds. In addition to OLDA and OMe-OLDA we investigated the ventilatory effects of 3′-O-oleoyl-N-oleoyl-dopamine (OL-OLDA), an ester of oleic acid and OLDA at its 3′ hydroxyl group of the catechol ring (Fig. 1c). The premise for investigating OL-OLDA was that the compound could be cleaved after passing into the brain by the omnipresent esterases, yielding bioactive OLDA. The potential of biogenic esters to act as prodrugs has been reported (Redasani and Bari 2016).

2 Methods

2.1 Animals

The study was approved by the Fourth Local Ethics Committee for Animal Experimentation in Warsaw, Poland. A total of 20 male Wistar rats, 11–12 weeks old, weighing 280–300 g were used in the study. The animals were kept individually in cages on a 12 h light-dark cycle, temperature of 21 ± 2 °C, and humidity of 50–60%, with commercial rodent chow and water *ad libitum*. They were randomly divided into three groups receiving equimolar doses of respectively OLDA (40 mg kg^{-1}, n = 7), OMe-OLDA (40 mg kg^{-1}, n = 7) and OL-OLDA (60 mg kg^{-1}, n = 6).

2.2 Ventilatory Measurements and Experimental Procedures

Ventilation and its responses to acute hypoxia were measured in a whole-body flow-through plethysmograph consisting of a recording and reference chamber (model PLY3223; Buxco Electronics, Wilmington, NC). Temperature in the recording chamber was maintained at 21 °C throughout the experiment. Pressure difference between the chambers was measured with a pressure transducer. The differential pressure signal was amplified and integrated by Biosystem XA software (Windows SFT3410 v2.9; Buxco Electronics). After a 30-min period of acclimatization to the body box environment, rats' basal ventilation was recorded in room air. Afterward, the inhalation air was switched to hypoxic mixture of 12% or 8% O_2 in N_2, randomly applied. The gas equilibrium in the chamber was achieved within 40 s. Hypoxic tests were of 3-min duration and were poikilocapnic. Bias flow at a rate of 2.5 l min^{-1} *via* a flow pump reservoir system (PLY1020, Buxco Electronics) was used to between the hypoxic tests to counteract the CO_2 build-up in the recording chamber. For recovery, a 20-min room air breathing was applied between the tests.

The OLDA, OMe-OLDA, and OL-OLDA compounds were synthesized at the Faculty of Chemistry of Warsaw University in Poland and generously donated for this study by Z. Czarnocki and. P. Roszkowski. All substances were dissolved in 0.3 ml DMSO (Sigma-Aldrich; Poznan, Poland) *ex tempore* and each solution was injected intraperitoneally at the end of the last normoxic recovery from the control hypoxic

runs. Thirty minutes after the injection the hypoxic tests were reinvestigated in like manner.

2.3 Data Elaboration

Airflow was recorded breath-by-breath. The integrated signal yielded tidal volume (VT; ml) and breath frequency (f; breaths min^{-1}), the product of which was a surrogate of minute ventilation (VE; ml min^{-1}). There were two time points of major interest during the hypoxic course which were taken for data comparisons: peak response at 30 s from onset of hypoxic measurement and hypoxic depressant nadir, ending the hypoxic exposure at 180 s. These points were compared with the baseline values preceding each hypoxic run.

Data are presented as means ±SE. VT and VE were normalized to kg of body weight to enable the unbiased comparison of values for different datasets. Comparisons within the groups were made with the non-parametric Wilcoxon test, and between groups with the Mann-Whitney U test. A $p < 0.05$ was set to define statistically significant differences.

3 Results

The profile of HVR was always typically biphasic, with a sharp ventilatory augmentation followed by a gradual fall-off. All three compounds investigated showed an inhibitory, albeit with some differences in strength, effect on both normoxic and hypoxic ventilation. The numerical ventilatory data for each compound for both 12% and 8% hypoxia are given below and the HVR profiles for the stronger 8% hypoxic stimulus in OLDA, OMe-OLDA, and OL-OLDA-treated rats are displayed in Fig. 2.

3.1 Ventilatory Effects of OLDA

In the OLDA-treated rats, baseline normoxic ventilation decreased from the control 723.9 ± 53.0 to 502.5 ± 22.0 ml min^{-1} kg^{-1} after OLDA, i.e., by about 31% ($p < 0.05$). The decrease was mostly mediated by a significant drop in breathing frequency (Fig. 2a).

Concerning the HVR, OLDA decreased the peak ventilatory augmentation, from the control 1335.8 ± 98.8 to 856.6 ± 73.7 ml min^{-1} kg^{-1} in 12% hypoxia, i.e., by 36%, and from 2279.0 ± 237.8 to 1338.3 ± 49.5 ml min^{-1} kg^{-1} in 8% hypoxia, i.e., by 41% ($p < 0.05$ for both). The effect was due mostly to a drop in frequency in the OLDA-treated rats, from 110.2 ± 9.8 to 85.7 ± 9.0 breaths min^{-1} and from 145.1 ± 11.6 to 106.7 ± 5.6 breaths min^{-1} in 12% and 8% hypoxia, respectively ($p < 0.05$ for both). Tidal volume also tended to drop in 12% hypoxia after OLDA, from the control 12.8 ± 1.3 to 10.3 ± 0.6 ml kg^{-1}; the drop assumed significance in the stronger 8% hypoxia, from 16.1 ± 1.3 to 13.0 ± 0.6 ml kg^{-1} ($p < 0.05$).

OLDA deepened the late HVR suppression, from the control 1165.2 ± 121.3 to 644.5 ± 35.5 ml min^{-1} kg^{-1} after OLDA in 12% hypoxia and from 2014.1 ± 246.3 to 1125.3 ± 73.2 ml min^{-1} kg^{-1} in 8% hypoxia, respectively ($p < 0.05$ for both). Thus, ventilation was lower in the hypoxic inhibitory phase by about 42% and 40% in 12% and 8% hypoxia, compared with the respective levels before OLDA treatment. This effect was due to reductions in both frequency, from 118.7 ± 9.9 to 76.5 ± 4.7 breaths min^{-1} and from 149.6 ± 15.7 to 112.5 ± 5.5 breaths min^{-1}, in 12% and 8% hypoxia, respectively ($p < 0.05$ for both) and tidal volume, from 9.9 ± 0.8 to 8.7 ± 0.4 ml kg^{-1} and from 13.5 ± 0.6 to 10.4 ± 0.5 ml kg^{-1} in 12% and 8% hypoxia, respectively ($p < 0.05$ for both).

3.2 Ventilatory Effects of OMe-OLDA

OMe-OLDA also decreased ventilation and its responses to hypoxia (Fig. 2b). Normoxic ventilation decreased from the control 831.6 ± 105.1 to 710.0 ± 72.2 ml min^{-1} kg^{-1} after OMe-OLDA ($p < 0.05$). This about 15% decline was mediated by a drop in breathing frequency from 98.0 ± 7.1 to 81.5 ± 5.9 breaths min^{-1}

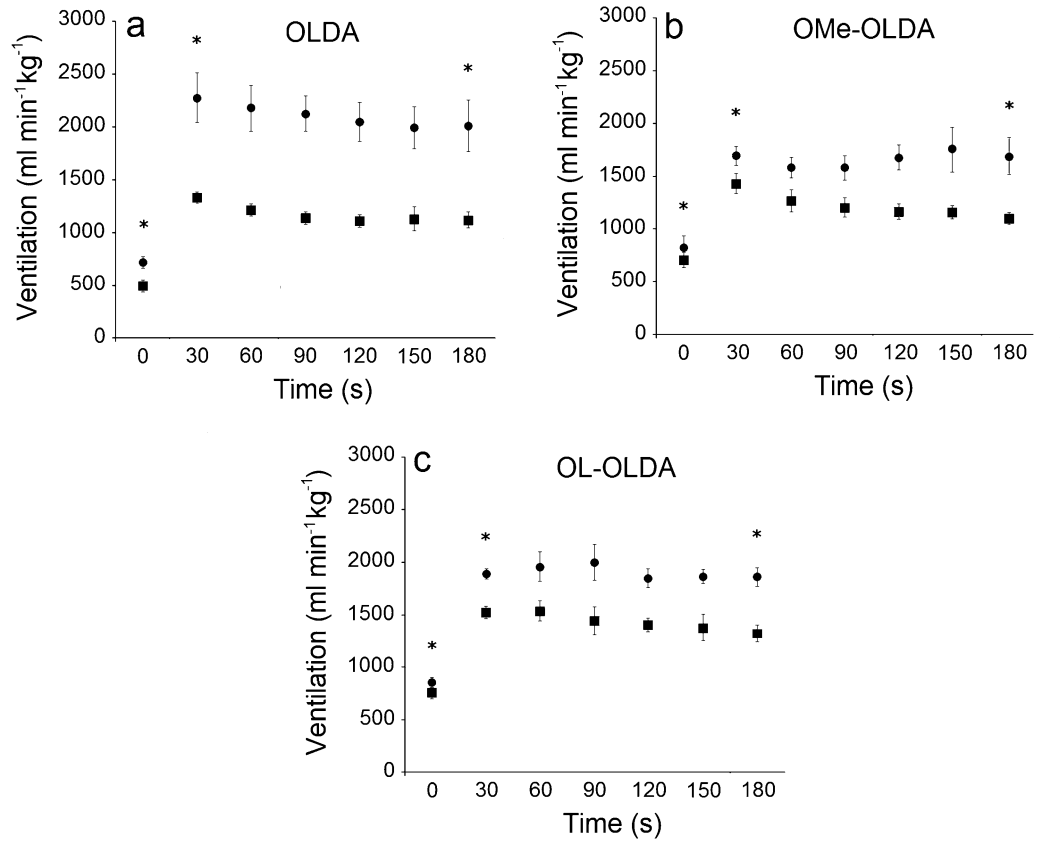

Fig. 2 Profiles of ventilatory responses to 8% hypoxia before (*circles*) and after (*squares*) treatment with N-oleoyl-dopamine (OLDA) (**a**), 3′-O-methyl-N-oleoyl-dopamine (OMe-OLDA) (**b**), and 3′-O-oleoyl-N-oleoyl-dopamine (OL-OLDA) (**c**). *$p < 0.05$ *vs.* corresponding control level before compounds administration at the three time points of major significance: normoxic baseline level, peak hypoxic augmentation at 30 s, and hypoxic suppressant nadir at 180 s from hypoxic stimulus induction

($p < 0.05$) rather than tidal volume that barely changed from 8.4 ± 0.5 to 8.8 ± 0.3 ml kg^{-1} after OMe-OLDA.

Concerning the HVR, OMe-OLDA decreased the peak ventilatory augmentation from 1347.0 ± 108.7 in the control to 1072.1 ± 76.8 ml min^{-1} kg^{-1} in 12% hypoxia and from 1698.3 ± 88.2 to 1443.8 ± 97.4 in 8% hypoxia. The decreases were by about 20% and 15% in 12% and 8% hypoxia, respectively ($p < 0.05$ for both). The effect was due mostly to a drop in frequency in the OMe-OLDA-treated rats, from 126.8 ± 4.1 to 104.9 ± 5.0 breaths min^{-1} and from 145.7 ± 4.8 to 119.9 ± 6.4 breaths min^{-1} in 12% and 8% hypoxia, respectively ($p < 0.05$ for both). Changes in tidal volume were insignificant, varying from 10.6 ± 0.6 to 10.3 ± 0.3 ml kg^{-1} and from 12.1 ± 0.7 to 12.0 ± 0.3 ml kg^{-1} in 12% and 8% hypoxia, respectively.

OMe-OLDA deepened the late HVR suppression from the control 1168.9 ± 96.6 to 991.2 ± 70.1 ml min^{-1} kg^{-1} in 12% hypoxia and from 1696.0 ± 174.9 to 1101.8 ± 55.0 ml min^{-1} kg^{-1} in 8% hypoxia ($p < 0.05$ for both). Thus, ventilation was lower in the suppressant phase by about 25% and 35% in 12% and 8% hypoxia, compared with the respective levels before OMe-OLDA treatment. This effect was due to reductions in frequency, from 132.1 ± 7.4 to 112.5 ± 4.3 breaths min^{-1} and from 163.9 ± 12.5 to 121.8 ± 6.2 breaths min^{-1}

in 12% and 8% hypoxia, respectively ($p < 0.05$ for both) and in tidal volume, from 9.2 ± 0.6 to 8.8 ± 0.4 ml kg^{-1} and from 11.0 ± 1.0 to 9.3 ± 0.3 ml kg^{-1} in 12% and 8% hypoxia, respectively; the decrease in tidal volume was significant in 8% hypoxia ($p < 0.05$).

3.3 Ventilatory Effects of OL-OLDA

Likewise, OL-OLDA decreased normoxic ventilation from the control 855.6 ± 26.4 to 764.1 ± 41.9 ml min^{-1} kg^{-1} ($p < 0.05$); i.e., by about 11% (Fig. 2c). The decrease resulted from about equal, albeit insignificant, downward trend in both frequency and tidal breathing.

Concerning the HVR, OL-OLDA also decreased the peak ventilatory augmentation from the control 1447.4 ± 45.1 to 1208.3 ± 78.6 ml min^{-1} kg^{-1} in 12% hypoxia and from 1885.9 ± 52.2 to 1530.4 ± 56.3 ml min^{-1} kg^{-1} in 8% hypoxia. The decreases were by about 17% and 19% in 12% and 8% hypoxia, respectively ($p < 0.05$ for both) and were driven by tidal volume decrease, from 10.5 ± 0.9 to 8.1 ± 0.6 ml kg^{-1} and from 11.9 ± 0.7 to 9.9 ± 0.9 ml kg^{-1} in 12% and 8% hypoxia, respectively ($p < 0.05$ for both). Breathing frequency remained grossly unchanged – 142.5 ± 10.0 *vs.* 152.2 ± 10.5 breaths min^{-1} and 164.9 ± 8.1 *vs.* 163.1 ± 8.4 breaths min^{-1}, before *vs.* after OL-OLDA in 12% and 8% hypoxia, respectively.

OL-OLDA deepened the late HVR suppression from the control 1543.6 ± 141.2 to 1054.2 ± 59.5 ml min^{-1} kg^{-1} in 12% hypoxia and from 1862.1 ± 88.5 to 1328.1 ± 81.5 ml min^{-1} kg^{-1} in 8% hypoxia ($p < 0.05$ for both). Thus, ventilation was lower in the suppressant phase by about 32% and 29% in 12% and 8% hypoxia, compared with the respective control levels before OL-OLDA treatment. A decrease in ventilation was here solely mediated by tidal volume that dropped from 10.2 ± 0.8 to 7.4 ± 0.5 ml kg^{-1} and from 10.4 ± 0.9 to 7.7 ± 0.3 ml kg^{-1} in 12% and 8% hypoxia, respectively ($p < 0.05$ for both), with inappreciable changes in breathing frequency.

3.4 Differences among the Ventilatory Effects OLDA, OMe-OLDA, and OL-OLDA

Differences among the ventilatory effects, exerted by the three N-acyl-dopamines investigated, at normoxic baseline and at peak and nadir of HVR, expressed as the percentage of the control level before compounds administration, are displayed in Table 1. OLDA decreased ventilation the strongest in both normoxia and hypoxia, affecting both tidal and frequency

Table 1 Ventilatory inhibition after administration of N-oleoyl-dopamine (OLDA), 3′-O-methyl-N-oleoyl-dopamine (OMe-OLDA), and 3′-O-oleoyl-N-oleoyl-dopamine (OL-OLDA) in normoxia (baseline) and in 8% hypoxia as the percentage of control level

		OLDA (n = 7)	OMe-OLDA (n = 7)	OL-OLDA (n = 6)
Minute ventilation	Normoxia	71.4 ± 5.9[a]	87.1 ± 3.9	89.6 ± 4.6
	Hypoxia peak	61.7 ± 5.7[ab]	84.2 ± 2.7	81.3 ± 2.6
	Hypoxia nadir	60.1 ± 7.6	65.3 ± 2.6	72.1 ± 5.8
Tidal volume	Normoxia	92.4 ± 4.9	105.5 ± 3.7	93.7 ± 4.6
	Hypoxia peak	83.6 ± 7.7	100.3 ± 3.9[c]	82.7 ± 3.5
	Hypoxia nadir	78.1 ± 5.2	84.4 ± 2.4	76.5 ± 4.8
Breathing frequency	Normoxia	79.6 ± 4.6	83.3 ± 2.1	97.4 ± 8.1
	Hypoxia peak	75.3 ± 5.3[b]	82.5 ± 3.9[c]	99.3 ± 4.4
	Hypoxia nadir	80.8 ± 10.6	74.7 ± 3.9[c]	93.3 ± 4.0

[a]$p < 0.03$ OLDA *vs.* OMe-OLDA
[b]$p < 0.03$ OLDA *vs.* OL-OLDA
[c]$p < 0.03$ OMe-OLDA *vs.* OL-OLDA

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components. Both OMe-OLDA and OL-OLDA decreased ventilation in a comparable manner, but significantly less than OLDA did ($p < 0.03$). OMe-OLDA failed to appreciably influence the tidal component of ventilation, while OL-OLDA did not much influence the frequency component. Overall, considering the smallest changes in ventilation as an optimal drug side effect, OMe-OLDA and OL-OLDA seemed to perform better than OLDA, albeit the differences in the inhibitory ventilatory effects among the three compounds were rather modest.

4 Discussion

The major finding of this study was that all three oleic acid derivatives of dopamine investigated displayed bioactivity related to ventilatory control. The compounds revealed an inhibitory effect on lung ventilation and its responses to the hypoxic stimulus, which is reminiscent of the action of dopamine proper. Further, the typically biphasic stimulatory/suppressant character of ventilatory responses remained unchanged, which shows that the innate mechanisms of chemosensory responses were not affected.

Dopamine, administered systemically, inhibits ventilation and its responses acting through D2 receptors in the carotid body, a sensory organ initiating the hypoxic chemoreflex. The effect, mediated by dopamine D2 receptors on carotid chemoreceptor cells, is fugacious due to dopamine's inability to cross the blood-brain barrier and rapid peripheral degradation. Therefore, it best comes into sight when bilateral carotid body neurotomy is performed (Ide et al. 1995; Zapata and Zuazo 1980; Nishino and Lahiri 1972) or a peripheral dopamine D2 antagonist, domperidone, is used, which causes a clear ventilatory stimulation (Zapata et al. 1996; Bee and Pallot 1995).

By contrast, the action of brain dopamine and its receptors, both localized in the neuronal network generating and regulating ventilation (Kline et al. 2002; Lawrence et al. 1995), is presumed to be stimulatory (Gargaglioni et al. 2008; Hedner et al. 1982). Central dopamine is deficient in neurodegenerative conditions, the exemplary of which is PD, which is one of the factors underlying ventilation and gas exchange disorders, possibly leading to chronic hypoxia (Onodera et al. 2000). Central deficiency of dopamine apparently predominates over carotid body dysfunction in the mechanisms of hampered ventilatory responses in PD (Bialkowska et al. 2016). However, dopamine delivery into the brain is hardly effective due to its rapid peripheral degradation and blood-brain barrier impenetrability for hydrophilic compounds. The pharmacotherapeutic methods trying to circumvent the issue, like using L-DOPA, are subject to doubtful benefit, side effects, and the gradually tapering action with time (Tambasco et al. 2011). Therefore, the rational for the present study was to investigate the representative compounds of a novel class of lipid derivatives of dopamine, N-acyl-dopamines, in search for alternative methods of dopamine delivery into the brain. To this end, we deemed it warranted to investigate the potential of oleic acid derivatives of dopamine to inhibit ventilation, an undesirable adverse effect in case of PD.

The three oleic acid derivatives of dopamine investigated, generally, did not greatly differ in the inhibitory action on ventilation. There was, however, a consistent impression that OMe-OLDA, which is a major metabolite of OLDA having a chemical structure resembling one of vanilloids (Zajac et al. 2014), showed the least inhibition of ventilation. There are some structural requirements for a compound that facilitate the binding to the dopamine D2 receptor; the most important dopamine receptor subtype in respiratory control (Gonzalez et al. 1994). One of them is the presence of catechol moiety with free hydroxyl groups. Another is the presence of a protonated amine group (Missale et al. 1998). It seems that none of the chemical structures of the compounds investigated fully fulfill these requirements as the amine group is blocked by the carboxyl group of oleic acid and

in case of OMe-OLDA one of the hydroxyl groups of catechol moiety is occupied by the methyl group. Nonetheless, both compounds act *via* a dopamine pathway as the ventilatory effects are abrogated by D2 receptor antagonism (Rekawek and Pokorski 2011; Zajac and Pokorski 2008).

Biological plausibility of endogenous synthesis of N-acyl-dopamines, first proposed by Pokorski and Matysiak (1998), has been confirmed in further studies. The flag compound N-oleoyl-dopamine (OLDA), with its synthesis and metabolism pathways akin to that of dopamine, has been identified in the mammal brains (Chu et al. 2003; Zajac et al. 2014). OLDA and OMe-OLDA apparently act as ligands for vanilloid type 1 (capsaicin) receptors (VR1), increasing calcium influx in VR1-transfected human embryonic kidney cell line *in vitro*, and causing hyperalgesia to heat *in vivo* (Almási et al. 2008; Chu et al. 2003; Huang et al. 2002). These studies also suggest the presence of a methyl group in the chemical structure of the compound's dopamine ring, the requirement fulfilled by OMe-OLDA, could be essential for an optimal interaction with receptors. Nonetheless, the exact physiological role of oleic acid derivatives of dopamine present in the brain remains elusive.

In a series of *in vivo* rat studies we have previously described a dopamine-like activity of exogenously administered OLDA, consisting if increased locomotion and myorelaxation. These effects are antagonized by haloperidol, a blood-brain barrier penetrating dopamine D2 receptor antagonist, suggesting a central origin of action (Konieczny et al. 2009; Przegalinski et al. 2006). Likewise, OMe-OLDA has a strongly expressed dopamine-like activity consisting of ventilatory inhibition (Rekawek and Pokorski 2011). N-acyl-dopamines can indeed penetrate the barrier and stay there in a stable form for a time (Zajac et al. 2006, 2012, 2014; Pokorski et al. 2003), raising a specter of the use of the compound as prodrugs carrying dopamine over to the brain in the neurodegenerative conditions, like PD, characterized by the loss of central dopamine. To fulfill this role it would be desirable to marginalize ventilation inhibition caused by N-acyl-dopamines as it could counter the benefits of dopamine reaching the brain.

OLDA also penetrates the carotid body, an organ that initiates the hypoxic chemoreflex (Pokorski et al. 2006). The organ is barrier-free and mediates the inhibitory effects of systemically administered dopamine through dopamine D2 receptors (Ward and Bellville 1982; Welsh et al. 1978). In the present study, N-acyl-dopamines were used in healthy rats. The compounds are liable to interact with D2 receptors in the carotid body to cause ventilatory inhibition, as the D2 antagonism reverses the inhibition (Rekawek and Pokorski 2011; Zajac and Pokorski 2008). In contrast, blockade of central VR1 receptors fails to affect OLDA-induced ventilatory inhibition (Zajac et al. 2010) and shows the opposing enhancing influence on ventilation in case of OMe-OLDA, a compound having a vanilloid-like structure (Rekawek and Pokorski 2011). In addition, the presence of VR1 receptors in the carotid body has never been substantiated. Actually, carotid body-mediated inhibitory ventilatory effects of N-acyl-dopamines could be mitigated by the central stimulatory dopamine elements. The situation seems different in the context of PD-like states where the central loss of dopamine predominates over the carotid body-mediated ventilatory control (Bialkowska et al. 2016). In such states dopamine delivered to the brain could enhance ventilation and its responses.

In conclusion, there are grounds to believe that oleic derivatives of dopamine might be good drug candidates for treatment of neurodegenerative disorders with dopamine deficiency. Considering the least changes in ventilation as an optimal side effect, OMe-OLDA with its chemical structure and properties facilitating the interaction with receptors seems particularly worthy of intensified exploration as a potential carrier of dopamine molecule into the brain. Oleic derivatives of dopamine offer an attractive promise as a novel pharmacotheraputic approach in brain dopamine deficiency. Alternative study

designs are needed to resolve the issue of the plausibility and benefit of the use of lipid derivatives of dopamine in neurodegeneration.

Conflicts of Interest The authors of this work are inventors of the European and US patents covering the medical applications of OMe-OLDA supported in part by the EU Innovative Economy grant POIG 1.3.2.-14–047/11.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 47–54
DOI 10.1007/5584_2017_68
© Springer International Publishing AG 2017
Published online: 6 July 2017

Intermittent Hypoxia and Unsaturated Aldehydes: Effects on Oral Epithelial Wound Healing

Katia Avezov, Lena Lavie, and Dror Aizenbud

Abstract

Obstructive sleep apnea (OSA) is a highly prevalent sleep breathing disorder characterized by intermittent hypoxia (IH), leading to blood hypoxemia, hypercapnia, and sleep fragmentation. Studies on the effects of OSA on oral epithelial tissue healing are limited. Smoking is considered a risk factor for OSA through the exposure to chemically active toxins, present in the smoke. Acrolein is the most chemically active unsaturated aldehyde, impairing a variety of biological processes. The aim of this study was to determine the effect of IH on oral epithelial tissue healing, with and without acrolein. HaCaT cells were wounded by a cross-scratch made in the cell cultures, considered as time zero. Then, cells were exposed to 28 IH cycles (5–20% oxygen) during 12 h using the BioSpherix OxyCycler-C42 system. Control cells were maintained in normoxic conditions or in sustained hypoxia (SH) (5% oxygen) for the same durations, after which all cells were maintained for additional 12 h in normoxia. The migrating abilities of cells were measured after 24 h by calculating the percent of the residual cross-scratch area. In parallel experiments, 25 μM acrolein were added to each treatment. We found

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that the scratch closure was the slowest under IH. After 24 h, the residual scratch area in the IH treated cells was 29.5 ± 13.4% of the initial area, while in normoxia and SH it was 9.2 ± 5.8% and 10.3 ± 11.3%, respectively ($p < 0.01$ for both *vs*. IH). Adding acrolein further attenuated the migratory ability in IH as compared to normoxia and SH. We conclude that IH delays the healing process of oral epithelial tissue by slowing the cells' migratory abilities. The healing might be further attenuated by chemically active unsaturated aldehydes such as acrolein.

Keywords
Acrolein • Healing process • Hypoxia • Obstructive sleep apnea • Oral epithelial tissue • Sleep fragmentation • Smoking

1 Introduction

Obstructive sleep apnea (OSA) is a highly prevalent breathing disorder in sleep, characterized by recurrent pauses in respiration (Lavie 2015; Lévy et al. 2015). These pauses result in intermittent changes in blood oxygen saturation, and are considered analogous to hypoxia and reoxygenation demonstrated in the condition characterized by ischemia and reperfusion. The accumulated evidence implicates the apnea-related multiple cycles of hypoxia/reoxygenation, termed intermittent hypoxia (IH), in promoting the formation of reactive oxygen species (ROS) and inducing oxidative stress. ROS are natural byproducts of mitochondrial oxygen metabolism, but hypoxia/reoxygenation and IH trigger mitochondrial dysfunction, resulting in increased ROS formation (Lavie and Lavie 2009; Lavie 2003). Also, leukocytes are activated in OSA patients and promote the production of higher amounts of ROS (Dyugovskaya et al. 2002), contributing to the inflammatory response that exacerbates the oxidative stress. OSA is also associated with cardiovascular disease and systemic disorders such as hypertension, hyperlipidemia, type 2 diabetes mellitus, and obesity, all of which results in the activation of inflammatory pathways, endothelial dysfunction, and atherosclerosis. It has been suggested that the clustering of these comorbidities with OSA is largely mediated through oxidative stress (Lavie and Lavie 2009).

Recent evidence points to the possible association of OSA with acquired tissue inflammation in the oral environment such as periodontitis; a disease affecting the tooth supporting tissues. Accordingly, a four-fold increased prevalence of periodontitis has been demonstrated in patients with OSA (Gunaratnam et al. 2009). Since both OSA and periodontitis are considered to be associated with systemic inflammation and cardiovascular morbidity, an association between these two disorders might be considered. Additionally, a very high prevalence (76%) of OSA is documented among patients awaiting surgical intervention for malignancies of the oral cavity and oropharynx (Payne et al. 2005). Presumably, common etiologic factors, such as cigarette smoke or oral cancer could play a role in the predisposition to OSA. Alternatively, the oxidative stress incurred by OSA may promote the development of inflammatory and cancerous pathologies in the oral cavity (Almendros et al. 2012). Along this line, increased mortality from cancer is also documented in patients with OSA (Nieto et al. 2012).

Cigarette smoking (CS) is considered as a risk factor for a wide range of disorders which overlap with OSA co-morbidities, such as cardiovascular disease and atherosclerosis, and is strongly

associated with oxidative stress and inflammatory cell activation as well. Ample studies have demonstrated that all oral cavity tissues are affected by CS, including the teeth, mucosa, salivary glands, and the saliva. The damage ranges from a simple tooth staining and inflammatory conditions to oral cancer (Johnson and Guthmiller 2007; Reibel 2003; Blot et al. 1988). CS components inducing oxidative stress include oxygen and nitrogen free radicals as well as aldehydes. The gas phase of CS contains more than 10^{15} free radicals *per* puff while the particulate phase contains more than 10^{17} free radicals *per* gram (Swan and Lessov-Schlaggar 2007). Moreover, cigarette smoke constitutes a major environmental source of human exposure to α, β-unsaturated aldehydes (e.g., acrolein, crotonaldehyde) capable of protein carbonylation that leads to protein dysfunction and increase in oxidative stress. High chemical reactivity of these aldehydes is due to a double bond reacting with -SH (thiol) groups of proteins in a Michael addition reaction characterized by a nucleophilic addition of a carbanion or another nucleophile to an α,β-unsaturated carbonyl compound (Grimsrud et al. 2008; Nagler et al. 2000). In this reaction aldehydic carbonyls are attached to a protein and induce structural alterations.

A synergistic effect has been suggested between cigarette smoking and sleep apnea on some of the biochemical cardiovascular risk markers such as C-reactive protein (CRP), triglycerides, and a decrease in high-density lipoprotein (HDL) cholesterol (Lavie and Lavie 2008). The synergism might be harmful to OSA patients who smoke, since cigarette smoke may act as an independent risk factor for OSA (Kashyap et al. 2001). Therefore, the epithelial integrity in the oral cavity is crucial for protecting it from external physical and microbial damages. Studies investigating the effects of OSA and IH on the oral cavity tissues are presently scarce. The purpose of the current study was to assess the healing abilities of oral epithelial cells under IH and to determine the potential synergism between the two oxidative stress inducers: IH and CS on epithelial wound healing by using acrolein, a CS smoke component.

2 Methods

2.1 Cell Culture

The HaCaT keratinocyte cell line was acquired from the Cell Lines Service (CLS; Eppelheim, Germany). HaCaT are keratinocytes that spontaneously transform from histologically normal human skin. The line is referred to as immortal (>140 passages). It maintains a full differentiation capacity and is non-tumorogenic (Boukamp et al. 1998). These cells are widely used (Ge et al. 2012) as a model for epithelial tissue studies, including oral epithelium, due to their high proliferation rate. The cells were cultured in Nunclon 24-well plates and incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal calf serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C humidified atmosphere containing 5% CO_2. When the cells reached 90% confluency, a cross-scratch was made in the middle of the well with a sterile 100 μL cone. The detached cells were removed by changing the culture medium and this scratched area was determined as time zero. The plates were immediately placed in the various oxygen chambers according to the protocol below. The migrating ability of cells was measured after 12 and 24 h by calculating the percent of the residual cross-scratch area.

2.2 *In Vitro* Intermittent Hypoxia Protocol

The cells were exposed to 28 IH cycles ranging from 5–20% oxygen during 12-h using the BioSpherix OxyCycler-C42 system (Redfield, NY) as previously described (Dyugovskaya et al. 2008). Control cells were maintained in a chamber with normoxic conditions or in

sustained hypoxia (SH) at 5% constant oxygen concentration for the same duration. Then, all the cells were transferred and maintained at normoxic conditions for additional 12 h (total 24 h) and the residual scratch area was determined again (Berger et al. 2013).

2.3 Exposure of Cell Cultures to Acrolein

Cell cultures were incubated with 25 μM acrolein (Sigma-Aldrich; St. Louis, MO), a content reported to be present in a mainstream smoke of one 2R1 University of Kentucky reference cigarette (O'Neill et al. 1994). These cigarettes with a known chemical content are used as standards for non-clinical investigations by tobacco manufacturers, contract and government laboratories, and academic institutions. Accordingly, in separate experiments, 25 μM acrolein were added to cell cultures at time zero, and then exposed to IH, SH, or normoxic conditions, as described above.

2.4 Image Stitching and Analysis

For high resolution whole-well imaging, the entire 24-well plate was photographed using the Zeiss Axio Observer Microscope (Carl Zeiss MicroImaging GmbH; Göttingen, Germany) equipped with a sensitive Hamamtsu Orca R2 camera (Hamamatsu Photonics; Welwyn Garden City, Hertfordshire, UK). The automated image overlap feature of the instrument was used at 5% overlap. In this way, the entire well and the scratch area of interest were imaged. Post-acquisition image files were automatically stitched, generating one image for each well.

3 Results

As illustrated in Fig. 1a and b, the scratch closure was the slowest under exposure to IH. After 24 h, the residual scratch area in the IH treated cells was 29.5 ± 13.4% of the initial area ($p < 0.05$), whereas in normoxia and SH it was 9.2 ± 5.8% and 10.3 ± 11.3%, respectively ($p < 0.01$ for both *vs.* IH). As illustrated in Fig. 2a and b, acrolein alone (acrolein + normoxia) attenuated the scratch closure; the residual scratch area was 84.5% and 33.4% of the initial area after 12 h and 24 h, respectively, whereas only 9.1% residual scratch area remained after 24 h in the normoxic control without acrolein. Combined treatment of acrolein and IH further attenuated epithelial cell closure. In the cells exposed to IH and acrolein, 91.8% and 53.5% of the initial scratch area remained after 12 h and 24 h, respectively. However, in the cells exposed to SH and acrolein, the residual scratch area was only 26.3% after 24 h (Fig. 2).

4 Discussion

In the current study we investigated the effects of various hypoxic treatments (IH and SH) and a component of CS – acrolein – on the migratory abilities of HaCaT keratinocytes. These cells are used as a model for oral epithelial wound healing in response to injury. The cells exposed to 28 IH cycles during the first 12 h showed the slowest scratch closure abilities compared with the cells exposed to SH or normoxia. After 24-h, the residual scratch area in the IH treated cells was 29.5 ± 13.4% of the initial area, whereas in normoxia and SH it was 9.2 ± 5.8% and 10.3 ± 11.3%, respectively. However, in response to a combined treatment with IH and acrolein a further decrease in the closure response was noted. After 24 h, 53.5% of the initial scratch area was still evident. The migratory abilities of HaCaT keratinocytes in the scratch areas might represent wound healing properties. Thus, intermittent hypoxia alone delayed wound healing; the effect was enhanced when hypoxia was combined with cigarette smoke. Comparable results were previously reported in a study where endothelial cell repair *in vitro* was delayed by the addition of OSA patients' serum compared with non-OSA serum (Briançon-Marjollet et al. 2014). Prolonged wound healing engenders the oral cavity to

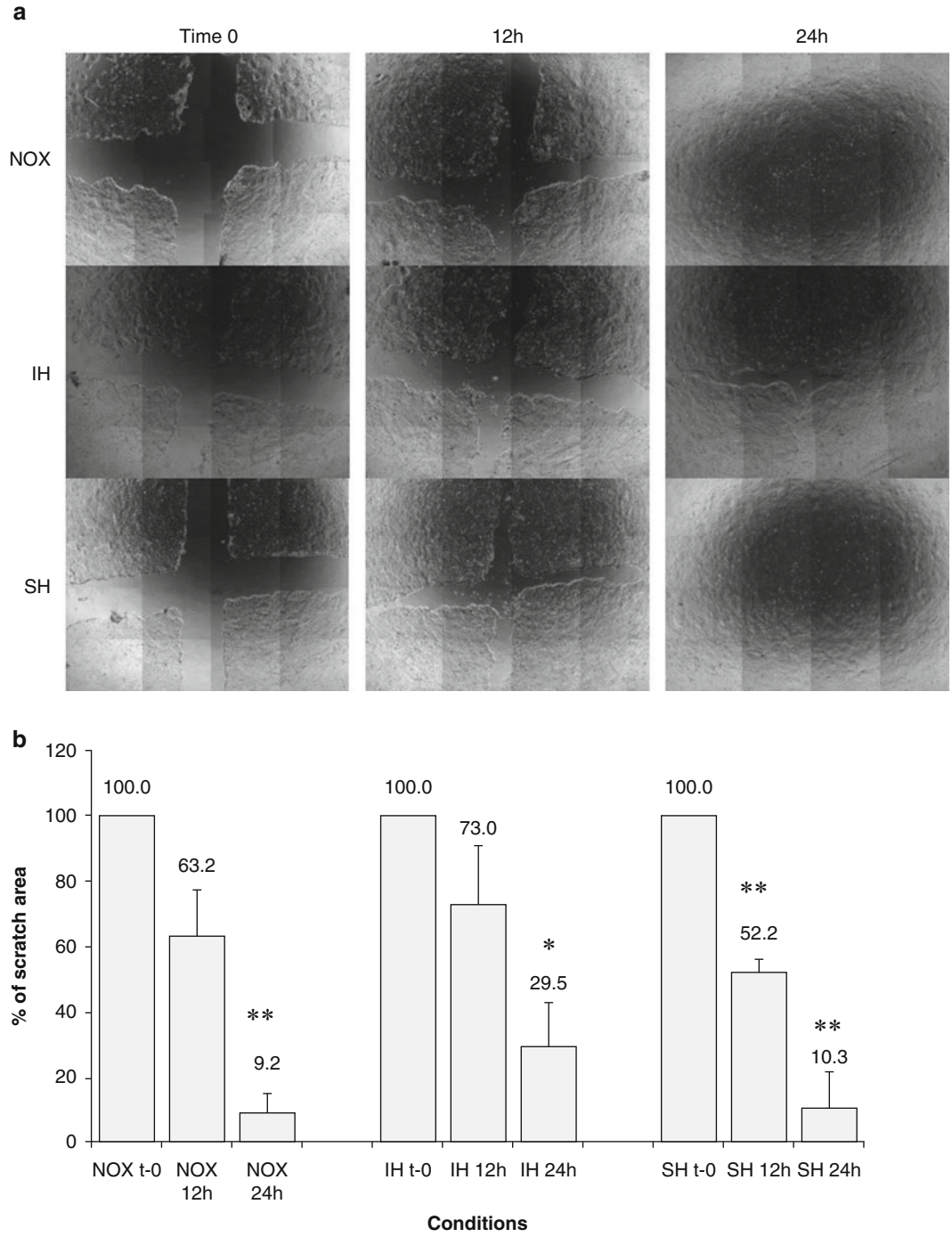

Fig. 1 Cross-scratch made in the middle of the well at time 0. HaCaT cells migratory abilities were measured after 12-h exposure to normoxia (NOX), intermittent hypoxia (IH), and sustained hypoxia (SH). Then, all cells were transferred to normoxia for additional 12 h and the remaining percentage of scratch area was determined (24 h total). (**a**) Representative whole-well images of the cross-scratch under NOX, IH and SH at time 0 (baseline), 12, and 24 h. (**b**) Quantitative representation of cell migrating abilities in the three experimental conditions and two time points outlined above. The percent of the residual cross-scratch area was measured 12 and 24 h after the initial scratch; data are means ±SD. *$p < 0.05$, **$p < 0.01$ in 12h and 24h vs. t-0 in each treatment condition (NOX, IH, SH)

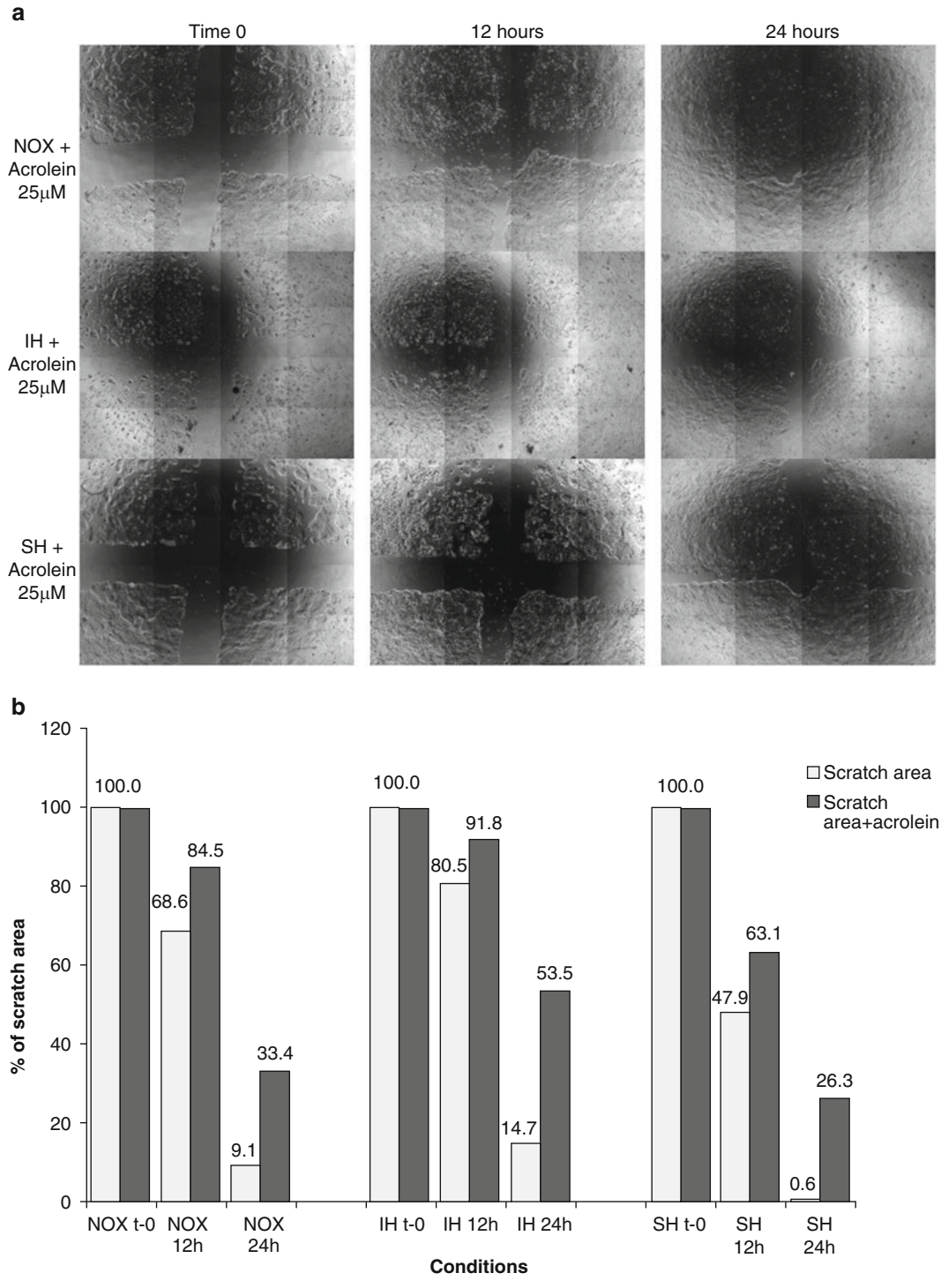

Fig. 2 Cross-scratch made in the middle of the well at time 0. Acrolein (25 μM) was added to HaCaT cell cultures. Cells migratory abilities were measured after 12-h exposure to normoxia (NOX), intermittent hypoxia (IH), and sustained hypoxia (SH). Then, all cells were transferred to normoxia for additional 12 h and the remaining % of scratch area was determined (24 h total). (**a**) Representative whole-well images of the cross-scratch with acrolein (25 μM) under NOX, IH, and SH at time 0 (baseline), 12, and 24 h. (**b**) Quantitative representation of cell migrating abilities in a representative experiment in the presence of acrolein in the three experimental conditions and two time points outlined above. The percent of the residual cross-scratch area was measured 12 and 24 h after the initial scratch

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potential threats particularly in smokers, who are exposed to an endless number of smoke toxins and oxidative stress. In a previous study we have shown that the addition of acrolein alone increases HaCaT intracellular protein carbonylation and oxidative stress and lowers glutathione (GSH) level, while N-acetylcysteine (NAC) decreases protein carbonylation and oxidative stress in these cells (Avezov et al. 2014). In the current study, acrolein also decreased the cells migratory abilities with a further attenuation observed in combination with acrolein and IH. Collectively, these data indicate that epithelial wound healing is largely affected by oxidative stress inducers such as IH and CS (Lavie 2015; Dyugovskaya et al. 2002).

OSA and IH are associated with a number of systemic disorders, many of which are mediated through oxidative stress as described for various organs and systems (Lavie 2015; Lavie and Lavie 2009). For instance, epithelial tissues in different organs are also affected by IH. The diameter of the seminiferous tubule and the height of the spermatogenic epithelium are significantly decreased in rats exposed to intermittent chronic hypobaric hypoxia compared with rats in normoxia (Cikutovic et al. 2009). In adult mice, IH increases alveolar surface area by stimulating lung growth (Reinke et al. 2011). Also, chemical hypoxia-induced injury in the HaCaT keratinocyte cell line is mediated by oxidative stress (Yang et al. 2011). Although the design of experimental hypoxia in those studies has been different from the present one, it is reasonable to assume that the oral cavity epithelium can also be affected by OSA and IH. Importantly, this notion is strengthened by a study showing that the prevalence of OSA in patients with chronic wounds exceeds the estimated prevalence of OSA in the general middle-aged population, which identifies a previously unrecognized population at risk for OSA.

The harmful effects of cigarette smoke and its constituents on the oral cavity are well known, while the studies investigating the effects of smoking on OSA or the presence of IH are limited. The current study emphasizes the previously proposed link between OSA and oral health (Gunaratnam et al. 2009). Moreover, it suggests a potential synergistic effect between OSA/IH and cigarette smoking on epithelial wound healing. Further studies are needed in order to better understand the pathways activated by IH, which may decrease the healing process of epithelial tissues.

Conflicts of Interest The authors declared no competing interests in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 55–63
DOI 10.1007/5584_2017_65
© Springer International Publishing AG 2017
Published online: 23 June 2017

Body Mass Disorders in Healthy Short Children and in Children with Growth Hormone Deficiency

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Abstract

The aim of the study was to determine the degree of adiposity and the incidence of body mass disorders, including abdominal obesity, in healthy short children and children with growth hormone deficiency. The study included 134 short children (height < 10th percentile) aged 7–15. In this cohort there were 63 (31 boys and 32 girls) children without diagnosed hormonal disorders and 71 patients (35 boys and 36 girls) with growth hormone deficiency. Basic somatic features were assessed and the study participants were categorized according to the percentage of body fat (% FAT), body mass index (BMI), and waist-to-height ratio (WHtR). We found that there were no significant differences in %FAT and the incidence of body weight disorders depending on gender or diagnosis. %FAT deficit was observed in 12–21% of the participants and underweight in almost every fourth child. Overweight involved 3–14% of the participants and obesity was diagnosed in isolated cases (0–3%); both were considerably lower compared to the estimates based on %FAT. Using the cut-off points of WHtR, abdominal adiposity was observed in 3–15% of the participants. In conclusion, quite a large number of short children (between 25 and 50%) are characterized by abnormal body fat or body mass index values. The results indicate a limited usefulness of BMI in evaluating the incidence of overweight and obesity in children characterized by a height deficit.

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Keywords
Adipose tissue • Body mass index • Central obesity • Growth hormone deficiency • Short children

1 Introduction

Growth disorders that result in child's short stature are a frequent reason for consultations with pediatric endocrinologists (Wudy et al. 2005). The majority of short children are diagnosed with constitutional delay of growth and puberty and familial or idiopathic short stature. However, after a period of necessary auxological observation and laboratory tests, only a small percentage of them require hormone therapy. According to a study by Dattani and Preece (2004), among children with a height of less than -2SD, only in approximately 14% does short stature have pathological etiology. It has also been demonstrated that only 5% of the children with a height lower than the 3rd percentile have previously unrecognized hormonal disorders or genetic syndromes responsible for short stature (Voss et al. 1992). Likewise, a population study evaluating the causes of short stature in children shows that only 5% of the 555 children analyzed (height below the 3rd percentile and growth rate below 5 cm *per* year) were diagnosed with hormonal disorders (Lindsay et al. 1994); the growth hormone deficiency (GHD) being one of the most frequent.

The congenital form of growth hormone deficiency occurs with the frequency of 1/4000–1/10,000 live births (Alatzoglou et al. 2014), whereas the acquired form accounts for approximately one-third of the patients with GHD. Data published in Poland demonstrate that children with GHD represent approximately 30% of patients reporting short stature (Majcher et al. 2012) and they are all included in a free therapeutic program. The purpose of treatment with recombinant growth hormone is to achieve an adult height above the 10th percentile, to correct metabolic disorders associated with GHD, and to improve patients' quality of life (Högler et al. 2005; Ahmad et al. 2001).

The prevalence of overweight and obesity in the general population has been well documented (Ogden et al. 2016; Ng et al. 2014), but the evaluation of the degree of adiposity and the incidence of weight-to-height ratio disorders in children with a height deficit has been the subject of few studies. Existing research demonstrating an increased incidence of obesity among short persons concern mainly adults (Hermanussen et al. 2005; López-Alvarenga et al. 2003). The studies conducted in healthy short children and adolescents have focused primarily on the evaluation of the values of weight-to-growth indices (Bosy-Westphal et al. 2009; Thibault et al. 1993), the grounds for their application in evaluation of overweight and obesity (Bonthuis et al. 2013), and their relation to nutrition (Wudy et al. 2005). These studies consistently indicate lower values of weight-to-height indices in short participants compared to their peers of normal height. The incidence of underweight, overweight, and obesity, including abdominal obesity, in short children was not estimated clearly, except for the study by Bosy-Westphal et al. (2009). Studies on weight-to-height ratios of healthy short children have been based on small samples of quite a large age range (Thibault et al. 1993), or not very sharp cut-off points (exceeding the 10th percentile) for classification of short stature (Freedman et al. 2004a, b).

It is known that the level of growth hormone determines, among others, normal metabolism. Hormone deficiency results in adverse consequences in adulthood such as obesity and a related increase in adipose tissue, particularly in the abdominal area, and a decreased muscle mass (Rasmussen 2010). Further, increased visceral body fat is associated with a decrease in endogenous growth hormone secretion and this,

in turn, may lead to the increased accumulation of visceral fat (Stanley and Grinspoon 2015). It has been assumed that children with GHD are characterized by increased adipose tissue. However, the available literature does not provide a clear confirmation of such a phenomenon. A review by Shalet et al. (1998) has noted that children with GHD are characterized by increased adiposity, particularly in the thorax area, however no values of the percentage of body fat (%FAT) are indicated and no reference to relevant literature is made. The conducted studies have focused primarily on evaluating the effectiveness of therapy with growth hormone, among others, in the normalization of body composition in children with growth hormone deficiency (van der Sluis et al. 2002). In contrast to the studies conducted among adults, the prevalence of body weight disorders in children with GHD has not been evaluated at all. For this reason, the aim of this study was to determine the degree of adiposity and the incidence of body weight disorders, including abdominal obesity, in healthy children with a height deficit and in children with growth hormone deficiency.

2 Methods

2.1 Study Participants

The study was approved by the local Ethics and Clinical Research Committee and was performed according to the guidelines of the Declaration of Helsinki. The children and their parents were informed about the purpose and form of the study and gave their informed consent. The study was conducted at the Department of Pediatrics and Endocrinology of Warsaw Medical University in Poland before the commencement of possible hormone therapy. There were 134 children enrolled into the study, aged 7–15, with a height deficit, out of which 63 (31 boys and 32 girls) were characterized by short stature without diagnosed hormonal disorders or growth hormone deficiency. The remaining 71 children (35 boys and 36 girls) were diagnosed with growth hormone deficiency. The height deficit was defined as height below the 10th percentile for a given age and gender, and the cut-off points were adopted based on the standards for Warsaw children (Palczewska and Niedźwiecka 2001). There were no cases of children born with intrauterine growth restriction, while eight children were born prematurely with body weight corresponding to the duration of pregnancy.

2.2 Methodology

Measurements of somatic features were carried out by an anthropologist using standard methodology and equipment (Hall et al. 2007). The patients' height was measured with a stadiometer (Holtain Ltd.; Crymych, UK) with an accuracy to 0.1 cm. Their body mass was estimated using a body composition analyser TBF-300A (Tanita Corp., Tokyo, Japan) with an accuracy to 0.1 kg, whereas the thickness of two skinfolds (above the triceps brachii and below the scapula) was measured with a Harpenden skinfold calliper (Holtain Ltd). Waist circumference was measured half-way between the 10th rib and the iliac crest using the anthropometric tape. Body mass index (BMI) and waist-to-height ratio (WHtR) were calculated for each studied child. The percentage of body fat (%FAT) was estimated according to Slaughter's equation (Slaughter et al. 1988).

Using reference values developed for the Polish population and the proposed cut-off points for %FAT and BMI, study participants were categorized by their degree of adiposity and weight-to-height ratios. The classification was performed using polynomial equations as functions of age computed for boys and girls, and BMI cut-off points corresponding to the World Health Organization's recommended values, i.e., BMI <18.5 – underweight, BMI >25 – overweight, BMI >30 – obesity. On this basis, children with decreased, normal, or increased adipose tissue and obesity were distinguished, and also children who, depending on their BMI, were characterized by underweight, normal weight, overweight, or obesity (for details see Tomaszewski et al. 2013). For a

more complete diagnosis, the prevalence of body weight disorders in the children studied was additionally evaluated using the International Obesity Task Force (IOTF) criteria. This evaluation included the cut-off points adjusted for age and gender in diagnosing underweight (Cole et al. 2007), and overweight and obesity (Cole et al. 2000).

The incidence of abdominal obesity was also evaluated using the standard WHtR criterion of $\geq$0.5, applied irrespective of gender and age (Browning et al. 2010). Additionally, the age- and gender-adjusted cut-off points for WHtR were used. These points, for the children aged 7 and 15, are 0.480 and 0.462 for boys, respectively, and 0.495 and 0.458 for girls, respectively (Stupnicki et al. 2013). Children with the excessive body fat content were further classified according to reference WtHR values defined as means $\pm$2SD. The WtHR value within normal limits was considered to indicate a relatively uniform peripheral fat distribution, while values above the upper limit indicated central (abdominal) fat deposition.

2.3 Statistical Elaboration

For data description, means $\pm$SD and percentages were used along with the respective measures of effect size (partial η^2 and Cramer's V for ANOVA and contingency tables, respectively). Normality and homogeneity of variances were tested using the Shapiro-Wilk and the Levene test, respectively. Differences in mean standardized values (Z-scores) of body fat percentage and BMI in healthy children with a height deficit and in children with growth hormone deficiency were evaluated using two-way analysis of variance ANOVA (gender $\times$ group). The incidence of a particular category of adiposity and WtHR value and of abdominal obesity in healthy short children and those with GHD was compared using the Chi-squared test. The significance of differences in the percentages obtained on the basis of different classifications of BMI was evaluated using a test for stratum weights. A p-value $<$0.05 defined statistically significant differences.

3 Results

Detailed data on somatic features and indices are displayed in Table 1. The mean values of %FAT ranged from 16.0% in healthy boys to 19.1% in girls with GHD, the mean BMI values were from 16.5 to 17.1 kg/m^2, and the mean waist circumference ranged between 56.6 and 59.9 cm. However, the analysis of variance showed no significant differences in the mean standardized values of body fat percentage and BMI depending on gender ($F_{1.125} = 1.38$; $p = 0.24$; partial $\eta^2 = 0.01$ and $F_{1.125} = 1.01$; $p = 0.32$; partial $\eta^2 = 0.008$, respectively) or on the study group ($F_{1.125} = 1.10$; $p = 0.30$; partial $\eta^2 = 0.009$ and $F_{1.125} = 0.56$; $p = 0.45$; partial $\eta^2 = 0.004$, respectively). The gender $\times$ group interactions

Table 1 Somatic features and indices of healthy short children (HSC) and those with growth hormone deficiency (GHD)

	HSC		GHD	
Variable	Girls(n = 32)	Boys(n = 31)	Girls(n = 36)	Boys(n = 35)
Height (SDS)	−2.20 ± 0.68	−2.05 ± 0.64	−2.57 ± 0.51	−2.51 ± 0.48
Body mass (SDS)	−1.29 ± 0.85	−1.22 ± 0.66	−1.21 ± 0.83	−1.38 ± 0.57
Body fat content (%)	18.3 ± 5.4	16.0 ± 6.0	19.1 ± 5.9	16.7 ± 5.9
Body fat content (SDS)	0.05 ± 2.63	−0.21 ± 2.64	0.82 ± 2.98	−0.01 ± 2.30
Body mass index (kg/m^2)	16.6 ± 3.5	16.5 ± 2.5	17.1 ± 3.8	17.1 ± 3.0
Body mass index (SDS)	−0.16 ± 1.56	0.41 ± 2.02	0.34 ± 1.65	0.36 ± 1.61
Waist circumference (cm)	57.6 ± 7.4	57.4 ± 6.0	56.6 ± 8.5	59.9 ± 7.9
Waist-to-height ratio	0.42 ± 0.04	0.43 ± 0.04	0.44 ± 0.05	0.44 ± 0.04

Data are means $\pm$SD

also failed to be significant for either variable outlined above ($F_{1.125} = 0.36$; $p = 0.55$; partial $\eta^2 = 0.003$ and $F_{1.125} = 0.84$; $p = 0.36$; partial $\eta^2 = 0.006$, respectively). The absence of significant differences depending on gender and hormonal disorders has been confirmed by the percentage of children assigned to particular categories of adiposity ($\chi^2 = 6.81$; $p = 0.66$; Cramer's $V = 0.13$) and BMI ($\chi^2 = 6.41$; $p = 0.70$; Cramer's $V = 0.12$ and $\chi^2 = 10.4$; $p = 0.32$; Cramer's $V = 0.15$ for the estimations based on Tomaszewski et al.'s (2013) and Cole et al.'s (2000, 2007) criteria. Despite quite large discrepancies in the estimated percentages of % FAT, particularly concerning the children with increased body fat and obesity, the differences were insignificant ($p \geq 0.29$).

The combined percentage of children with increased body fat content, but remaining below obesity, and with obesity ranged from about 13% in healthy short boys to 35% in girls with GHD (Table 2). Obesity alone was present in single cases, from 0% to 3%, in healthy short girls and boys, respectively, with the reversed percentage in GHD children. Likewise, the percentage of children who were overweight was not very high, amounting to about 6.5% in healthy short children and 8–14% in children with GHD. Concerning the incidence of body fat deficit, about 12% of girls and 21% of boys with GHD were assigned to this category. On the other hand, underweight was observed in a far greater number of children (16–31%) based on the cut-off points for BMI assessment, but it ranged from 19% to as much as about 44% when BMI was based on the IOTF criteria. Despite the substantial spread of data obtained with the two standards of BMI assessment, the differences failed to reach statistical significance ($p \geq 0.75$).

Using the cut-off points of WHtR, taking into account gender, age, and the percentage of body fat, abdominal obesity was observed in 9.7% of healthy short girls and 3.2% of short boys, and in 14.7% of girls and 9.1% of boys with GHD ($\chi^2 = 2.80$; $p = 0.42$; Cramer's $V = 0.14$). Somehow different, albeit insignificantly so ($p \geq 0.45$), estimates of the incidence of abdominal obesity were obtained using the commonly used WHtR ≥ 0.5 criterion. These values were 6.5% in healthy girls and boys and 8.3% and 11.4% in girls and boys with GHD, respectively ($\chi^2 = 0.71$; $p = 0.87$; Cramer's $V = 0.07$). Interestingly, after applying the IOTF criteria to all 11 children with abdominal obesity according to the WHtR ≥ 0.5 criterion, only two of them were categorized as obese, six as overweight, and three as having normal weight.

Table 2 Percentages of healthy short children (HSC) and children with growth hormone deficiency (GHD) stratified by the body fat percentage and the body mass index

	HSC				GHD			
	Girls (n = 32)		Boys (n = 31)		Girls (n = 36)		Boys (n = 35)	
Body fat content[a]								
Decreased	19.3		16.1		11.9		21.2	
Normal	61.3		71.0		52.9		60.6	
Increased (below obesity)	9.7		9.7		17.6		9.1	
Obesity	9.7		3.2		17.6		9.1	
Body mass index[b, c]	I	II	I	II	I	II	I	II
Underweight	31.2	43.7	16.1	19.4	25.0	36.1	25.7	25.7
Normal	62.5	50.0	74.2	74.2	63.9	50.0	60.0	65.7
Overweight	6.3	6.3	6.5	3.2	8.3	11.1	14.3	8.6
Obesity	0.0	0.0	3.2	3.2	2.8	2.8	0.0	0.0

I – percentages according to Tomaszewski et al.'s criteria (2013); II – percentages according to IOTF criteria (Cole et al. 2000, 2007); [a]inter-gender difference: $\chi^2 = 6.81$; $p = 0.66$; [b]inter-gender difference for estimation using criteria I: $\chi^2 = 6.41$; $p = 0.70$; [c]inter-gender difference for estimation using criteria II: $\chi^2 = 10.4$; $= 0.32$

4 Discussion

The existing studies on weight-height relationships mainly concern the estimates for the general population. Attempts to assess the incidence of body mass disorders in healthy children diagnosed with disorders of growth processes are scarce, while in children with GHD there is a complete absence of research on the topic. The present study attempted to fill this gap in the literature. Our results indicate a frequent incidence of body fat deficit in short children, ranging between 12–21%, with no significant differences between healthy short children and children with GHD. It is worth noting that in girls with GHD the mean standardized value of %FAT was relatively high (SDS = 0.82). Increased body fat or obesity was diagnosed in 35% of those girls, which to some extent confirms the common assumption about excess body fat in children with GHD. Nonetheless, the results obtained among boys with GHD do not fully confirm this assumption since they are comparable with those observed in healthy short boys and in population studies.

Considerably different characteristics were noted concerning the estimates made with BMI. Using the cut-off points for BMI, while taking into account the %FAT, on average, almost every fourth short child was diagnosed as underweight irrespective of the presence or absence of GHD. The use of the IOTF criteria for BMI showed these percentages were even higher, reaching 35% in girls with GHD and about 44% in healthy short girls. On the other hand, irrespective of the criteria used, overweight was diagnosed much less frequently, ranging from 3% to 14%, and obesity only sporadically. These findings are in line with the literature BMI estimates for children with short stature. Freedman et al. (2004b) have found that the incidence of overweight (BMI $\geq$95th percentile) varies between 0% and 14% among short boys (height $<$ 25th percentile) aged 5–8 and 12–14, respectively. Among girls, these percentages are 9% and 15%, respectively. Those observations have partially been confirmed in population studies in which the incidence of overweight or obesity is estimated at only 1–2% among the shortest children (height $<$ 20th percentile) aged 3–10, and at 5–9% for those aged 11 to 17 (Freedman et al. 2004a). In the German population, overweight (BMI $\geq$90th percentile) and obesity (BMI $\geq$97th percentile) have been found in about 7% of children and adolescents with a height below the 10th percentile, irrespective of gender (Bosy-Westphal et al. 2009). In that study, percentages of the shortest boys and girls with body mass deficit (BMI $\leq$10th percentile) were about 8% and 13% respectively, and were considerably lower than those observed in the present study. The incidence of overweight and obesity estimated with BMI was considerably lower among short-statured children in the present study than that reported in the general population of Polish children (Kułaga et al. 2011), which does not clearly confirm the assumption of increased adiposity in children with GHD. However, certain limitations of the use of BMI should be considered when attempting to draw inferences from the data. It has often been demonstrated that the diagnostic value of BMI as a measure of body fat or the risk of obesity-related diseases in children is insufficient (Freedman et al. 2005; Schaefer et al. 1998), particularly in short subjects (Lara-Esqueda et al. 2004). The results of studies conducted among girls with Turner syndrome (Tomaszewski et al. 2008) also indicate the incompatibility of the categorization made using the percentage of body fat (BIA method) and BMI, and a low usefulness of the latter in the evaluation of normal body weight in girls with a height deficit. It has also been indicated that BMI is moderately correlated with height which can result in the underestimation of BMI values in short children as compared to their peers of normal height (Wells 2001). Some authors suggest that in children with disorders of growth and, particularly, a height deficit, BMI should be related to height-age rather than calendar-age, when evaluating the incidence of obesity (Bonthuis et al. 2013; National Kidney Foundation 2009). The BMI

measurements should be complemented with additional methods of body composition evaluation.

Recently, WHtR has gained a considerable popularity in diagnosing abdominal obesity as it is considered a more reliable index of adiposity and associated risk of cardiovascular diseases and metabolic disorders compared to BMI (Brambilla et al. 2013; Savva et al. 2000). A fixed value, irrespective of gender, of WHtR $\geq$0.5 has been adopted as a criterion for abdominal obesity (Browning et al. 2010). Using this criterion, the present study demonstrates that abdominal obesity was present in 6.5% of healthy short children, and in 8.3% of girls and 11.4% of boys with GHD; the differences among these percentages did not reach statistical significance. The validity of applying WHtR in persons whose growth process is not yet complete and the use of the same WHtR value for both genders are questionable (Tybor et al. 2008). Therefore, we also evaluated the incidence of abdominal obesity using the cut-off points developed specifically for the Polish population, taking into account the percentage of body fat, age, and gender (Stupnicki et al. 2013). These estimates appear somehow different as abdominal obesity was diagnosed in 9.7% of healthy short girls and 3.2% of boys, and in 14.7% of girls and 9.1% of boys with GHD. It is noteworthy that out of the 11 children with abdominal obesity (WHtR $\geq$0.5) only two were classified as obese using the BMI and the IOTF criteria. This fact confirms, to an extent, limited usefulness of BMI in diagnosing obesity in children with a height deficit. It can be presumed that the incidence of abdominal obesity is comparable in healthy short children and those with GHD, but lower than that reported in population studies (Schröder et al. 2014; McCarthy and Ashwell 2006; Li et al. 2006). It is hard to reach final conclusions since, except one study of short adults (Velasquez-Melendez et al. 1999), WHtR has not been applied to estimate the incidence of abdominal obesity in short children. Neither has there been an attempt to assess the validity of WHtR in patients with a height deficit, particularly growing ones. Despite a limited patient sample in the present study, we believe the findings shed light on the relationship between adiposity and WHtR in children with a height deficit, including children with GHD. It should be kept in mind that children with short stature may have a predilection to obesity and the associated syndromes in adulthood (Bosy-Westphal et al. 2009). This is of particular concern in GHD as many of such patients struggle with obesity in adulthood (Rasmussen 2010). On the other hand, short stature also may be accompanied by underweight and the ensuing body weight deficit, which appears in every third short adult (Greco et al. 1995). Thus, it seems essential to maintain WHtR in a normal range throughout the child's development, aside from treating the cause of short stature in an attempt to achieve a taller height. In this context, emphasis should be placed on improving the child's eating and physical activities.

5 Conclusions

A considerable number of children with short stature are characterized by abnormal body fat or body mass index, which requires a thorough medical control to exclude coexisting chronic illnesses. The findings of the present study demonstrate that the distribution and size of adipose tissue and the weight-to-height ratio were comparable in healthy short children and children with growth hormone deficiency. The study failed to substantiate the presumption that growth hormone deficiency would be linked to increased body fat. The difference between the estimates based on the percentage of body fat and body mass index points to a limited usefulness of the latter assessment in evaluating the prevalence of overweight and obesity among children with a height deficit.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 65–72
DOI 10.1007/5584_2017_66
© Springer International Publishing AG 2017
Published online: 19 July 2017

Survival of Patients with Cystic Fibrosis Depending on Mutation Type and Nutritional Status

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Abstract

The purpose of the study was to evaluate the influence of nutrition and of the severity of mutation type on survival rate in cystic fibrosis (CF) patients. Data were longitudinally collected from 60 hospitalized adult CF patients, aged 18–50. The variables consisted of body mass index (BMI) ratio, Cole's BMI cut-off points, severity of mutation type, and survival rate of CF patients. We found that the mean BMI was strongly associated with the severity of mutation type and was significantly lower in patients with severe mutations of grade I and II. The mutation type significantly affected the patients' survival rate; survival was greater in patients with mild and undefined mutation types. The BMI and Cole's cut-off points also had a significant influence on survival rate. CF patients, who suffered from malnutrition and emaciation, had a shorter survival rate than those with proper nutritional status. In conclusion, the study findings confirmed a significant effect of nutritional status and of mutation type on survival rate of CF patients.

Keywords

Body mass index • Cystic fibrosis • Mutation • Nutritional status • Life expectancy

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1 Introduction

Cystic fibrosis (CF) is caused by a mutation in the *CFTR* gene (cystic fibrosis transmembrane conductance regulator), the consequence of which is incorrect structure and functioning of the CFTR protein (Mall and Hartl 2014). The mutation results in the elevated content of chloride ions in sweat, followed by multi-organ failure including pulmonary insufficiency, exocrine pancreatic insufficiency, and liver disease (Fanen et al. 2014). Molecular tests systematically carried out since 1990 have by far unraveled 2009 disease mutations (Cystic Fibrosis Mutation Database 2011). The impact of the mutation type on clinical expression of the disease is now one of the hot issues concerning CF. Thanks to the development of genetic testing of the *CFTR* gene, it is possible to detect the disease early on, already in newborns, which contributes to the rapid introduction of an appropriate treatment; thereby to the extension of patients' life. However, studies on the relationship between physical development, nutritional status, and mutation type in CF patients are still rather sparse. The influence on the patients' lifespan of these factors is of essential clinical importance. So far, it has been postulated that nutritional status could be a predictor of the length of CF patients' life (Simmonds et al. 2010; Sharma et al. 2001). According to Groleau et al. (2014), improvement of nutritional status of children with CF has a positive influence their development and metabolism, and increases the patients' energy resources. Mutation type, in turn, is recognized as one of the most important factors determining the survival rate. Severe mutations of grade I and II of at least one allele reduce survival of CF patients (de Gracia et al. 2005). Studies in adult patients with CF are rarely conducted. Hence, the aim of the present was to evaluate the effect of nutrition and of the severity of mutation type on survival rate of adult CF patients.

2 Methods

This study was approved by a local Ethics Committee and was conducted in accordance with the guidelines of the Declaration of Helsinki for Human Research. The research was longitudinal and was conducted between 2004 and 2015. Sixty patients, aged 19–50 (31 women and 29 men), hospitalized in the Department of Pulmonology, Allergology and Respiratory Oncology of Poznan University of Medical Science in Poland were enrolled into the study. In the 11-year study period, 14 patients died. Anthropometric measurements consisted of height measured to the nearest 1 cm and body mass to the nearest 0.1 kg. Body mass index (BMI) and Cole's cut-off points were used to assess the nutritional status (Cole et al. 2007). Based on BMI, the following groups were distinguished among patients: malnourishment (BMI $\leq$ 18.5), normal nutrition (BMI = 18.5–24.9), and overweight patients ($\geq$ 25). The undernourished patients were further stratified into emaciation (BMI $<$ 16), class I malnutrition (16 $\leq$ BMI $<$17), class II malnutrition (17 $\leq$ BMI $<$ 18.5).

Cystic fibrosis was diagnosed on the basis of on medical interview, clinical symptoms, and the elevated level of chloride in sweat. The diagnosis was confirmed with genetic testing. To systematize a host of known mutations, they were divided into three groups based on severity, with consideration given to the widely accepted mutation classification in the *CFTR* gene (Fanen et al. 2014; Welsh and Smith 1993). Accordingly, 40% of patients had severe mutations, 40% had mild mutations, and 20% had unspecified mutations (Table 1). Among severe mutations, delF508 mutation in both alleles occurred with the greatest frequency (10 persons). Among mild mutations, delF508 mutation in one allele with an unspecified mutation in the second allele occurred with the highest frequency (14 persons).

Table 1 Groups of patients in mutation classes of *CFTR* gene

Mutations	Consequences	Distinguished groups	n (%)
First class	Abnormal *CFTR* production	Severe mutations	
Second class	Abnormal post-translational process		24 (40)
Third class	Abnormal chloride channel regulation		
Fourth class	Dysfunction of chloride channel conductance	Mild mutations	24 (40)
	Reduction in *CFTR* protein efficiency		
Undefined	Unknown	Undefined mutations	12 (20)

Table 2 Stratification of cystic fibrosis patients according to the nutritional status

		n (%)
BMI (kg/m^2)	≤18.5	23 (38)
	18.5–24.9	33 (55)
	≥25.0	4 (7)
Cole's cut-offs	Emaciation	7 (11)
	Class II malnutrition	3 (5)
	Class I malnutrition	13 (22)
	Normal nutrition	37 (62)

BMI body mass index

Table 3 BMI in cystic fibrosis patients depending on mutation severity

Mutation category	n	Mean BMI (kg/m^2)
Severe	24	18.6 ± 3.1*
Mild	24	20.6 ± 3.6
Undefined	12	20.9 ± 1.9

Data are means ±SD. *BMI* body mass index; *p = 0.007 (Kruskal-Wallis test)

The following variables were used in data analysis: severity of mutation type, BMI, Cole's cut-off points, and the length of patient's life. The relationship between the genetic underpinnings of cystic fibrosis and nutritional status was defined using the Kruskal-Wallis test. The effect of the severity of mutation type and nutritional status on survival was determined with the Kaplan Meier method. Differences in the survival rate within the study groups were assessed with Chi-squared test for multiple samples. A p-value <0.05 defined statistically significant differences. Statistical elaboration was conducted with Statistica v10.0 commercial package (StatSoft; Tulsa, OK).

3 Results

The assessment of nutritional status, with both BMI and Cole's cut-off points, revealed that a significant proportion of CF patients were below the norm. Thirty eight percent of patients were underweight with BMI below 18.5 kg/m^2, while 55% stayed. The remaining 7% of patients were overweight. Using Cole's cut-off points, 12% of patients were classified as having emaciation, 22% were placed in class I, and 5% in class II malnutrition. These results are presented in Table 2.

Nutritional status of cystic fibrosis patients in the categories of mutation severity studied is shown in Table 3. The mean BMI fluctuated about the lower edge of the normal range in the group of patients with severe mutations and was significantly lower than those in patients with mild or unspecified mutations (p = 0.007).

The next stage of the analysis was to estimate the probability of survival. Survival analysis using The Kaplan-Meier plot shows that survival of CF patients remains at the level of 100% up to 19 years of age; afterwards it begins to decline gradually (Fig. 1). About 25% of patients died before 30 years of age, and another 25% of patients died before reaching 40 years. Thus, the period between 30 and 40 years of age should be considered critical concerning the survival of patients with cystic fibrosis. After 40 years of age, a sharp decrease in the probability of survival continues. In the present study group, only did five patients go over 40 years of age; three persons being still alive at the ages of 42, 43, and 50 at the time of writing up this article.

The probability of survival was different depending on the severity of mutation type (Chi^2 = 6.42, df = 2, p = 0.040). The

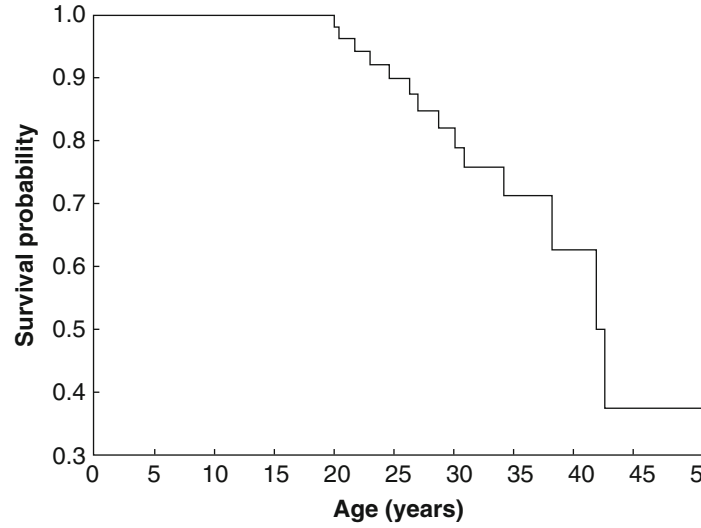

Fig. 1 Kaplan-Meier plot for age of patients with cystic fibrosis

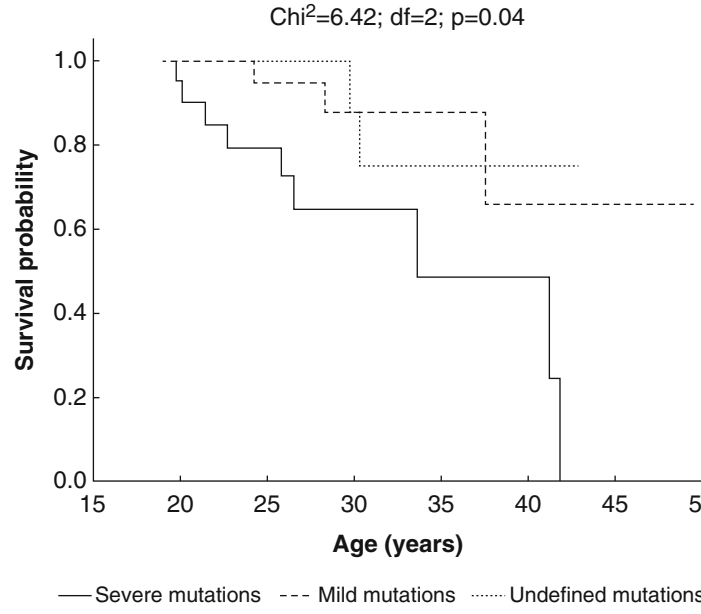

Fig. 2 Probability functions depicting age of patients with cystic fibrosis in categories of mutation

longest average survival was found in patients with mild and unspecified mutations, and the shortest in those with severe mutations (Fig. 2). Further, nutritional status assessed by BMI (Fig. 3) and by Cole's cut-off points (Fig. 4) significantly influenced the length of patient's life ($Chi^2 = 12.45$, df = 2, p = 0.002; and $Chi^2 = 30.98$, df = 3, p = 0.001, respectively). The probability of survival in malnourished patients was significantly lower than in those staying within the normal nutritional range. On the other side, survival rate in overweight patients was a 100%; none of those patients died during the observation time. The use of Cole's cut-off curve enabled the estimation of survival in the patients falling below the nutritional standard

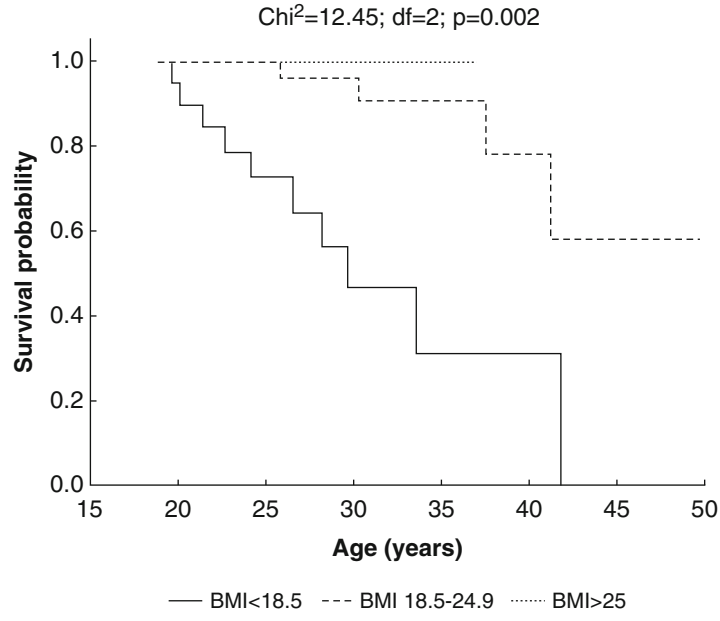

Fig. 3 Probability functions depicting age of patients with cystic fibrosis in categories of body mass index (BMI)

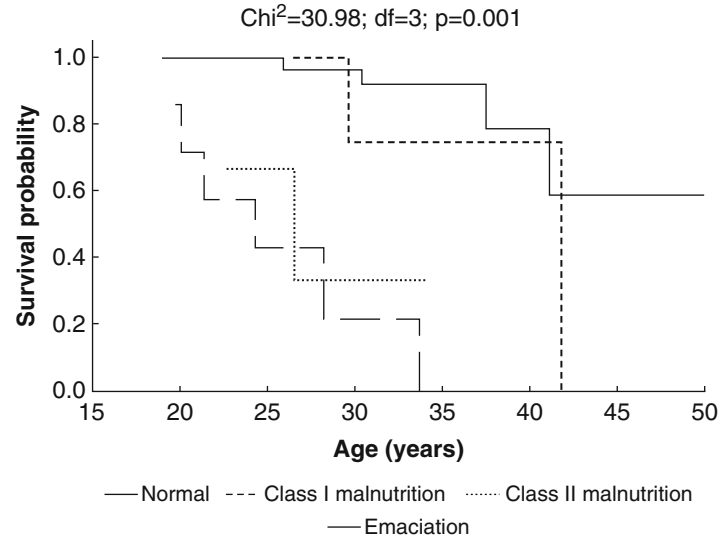

Fig. 4 Probability functions depicting age of patients with cystic fibrosis in categories of nutritional status

indicator. The average survival rates of patients with malnutrition class II and with emaciation were much lower than those of patients with malnutrition class I or staying within the norm. The lowest survival rate was found in the emaciated patients; none from this group lived to be 34 years old. The patients with malnutrition class I and those properly nourished lived much longer; reaching 40 and 50 years of age, respectively.

4 Discussion

The course of CF is complicated and is largely a consequence of genetics. Depending on the type of mutation, the disease can be mild or severe (Fanen et al. 2014). Severe type of mutation in the *CFTR* gene is associated, among others, with the deterioration of nutritional status (Dray et al. 2005; Panagopoulou et al. 2014). This relationship has been confirmed in the present study as patients with severe mutations had a lower average BMI than those with mild and unspecified mutations. Patients with severe mutations, more frequently exhibit pancreatic insufficiency (Ahmed et al. 2003), *Pseudomonas aeruginosa* acquisition (Lai et al. 2004), and reduced pulmonary function (Drumm et al. 2005). These sequalae contribute to a reduction in energy reserves, hampering patients' nutritional status and survival rate. The present study demonstrates that survival of CF patients with severe mutations may be lower by a decade or so compared to those with mild and unspecified mutations, which is consistent with the results of other studies (de Gracia et al. 2005; Lai et al. 2004). The implication is that an early detection of CF and determination of its severity are essential for the development of appropriate forms of therapy to achieve the best possible life expectancy.

Apart from the type and severity of mutation, the course of CF is influenced by environmental factors, including proper diet, and by pharmacotherapy chosen (Castellani et al. 2008). According to CF Genotype-Phenotype Consortium (1993), the type of mutation may be a good predictor of pancreas function, but it would not have a prognostic significance for lung function. Thus, respiratory failure in the course of CF largely depends on environmental factors. Early interventions concerning the growth and nutritional status of children with CF may improve lung function (Konstan et al. 2003). It is just warranted to consider how nutritional status affects the patient survival in CF.

Many studies carried out so far confirm the issue of malnutrition among patients with CF (Panagopoulou et al. 2014; Kosinska et al. 2008; Dray et al. 2005; Anthony et al. 1999). The present findings demonstrate that nutritional status of a large number of CF patients was below the norm, which hampers survival probability. The average survival probability varied depending on nutritional status. The lowest survival concerned patients with BMI below the norm, their survival did not exceed 42 years of age. In these patients, the shortest survival, not exceeding 35 years of age, concerned patients with Class II malnutrition, who showed the signs emaciation. By contrast, survival probability of overweight patients remained plainly unchanged at level 1.0 (Fig. 3), meaning no patients died in this group. These results confirm the previously conducted studies showing that nutritional status in CF patients is associated with survival and may be its independent predictor (Dodge and Turck 2006; Sharma et al. 2001). Malnutrition and emaciation is associated, among others, with the deterioration of lung functions (Stephenson et al. 2013; Gozdzik et al. 2008), reduced bone mineralization (King et al. 2005), and a reduced length (Sharma et al. 2001) and quality of life (Shoff et al. 2013). According to Hollander et al. (2014), BMI below 18.5 kg/m^2 reduces the chance of survival in patients who are candidates for lung transplantation. In turn, better nutritional status improves lung function (Woestenenk et al. 2013; Konstan et al. 2003).

Improved nutritional status improves patients' quality of life and defense abilities of the organism, and contributes to the regression of clinical symptoms of CF, which increases the survival rate. Nutritional status, alongside the severity of mutation, is an important predictor of survival in CF patients and its evaluation may be helpful in identifying critical moments in patients' lives related to reduced energy resources or body emaciation. However, it should be considered that nutritional status in CF patients may be affected by clinical consequences of the disease such as the multi-organ failure, foremost including the lungs. According to Pencharz and Durie (2000),

body weight of patients is maintained within the normal range until the occurrence of a significant deterioration of lung function. Malnutrition is thus also a reflection of the biological status of patients, while its predictive role concerning the patients' survival remains unchanged.

5 Conclusions

Survival in cystic fibrosis depends on the severity of mutation in the *CFTR* gene and on nutritional status of patients. The survival probability in case of severe mutations is lower than that in mild mutations. In turn, survival probability in patients whose nutritional status is within the normal range is higher than that in malnourished patients. In addition, survival varies depending on the extent of malnutrition.

Cystic fibrosis is characterized by a diversified clinical expression, which results from a multitude of *CFTR* mutations, environmental and individual health status, and medical care. The interrelationship between physical development and nutritional status of patients and the severity of mutation type may help predict the disease course, the proper selection of therapy, all of which affects the life expectancy. Further explorations are needed on the complex impact of biological factors on survival of patients with cystic fibrosis.

Acknowledgments This work was funded by grant GDWB-01/2016 Research Project founded by the Dean of the Faculty of Biology of The Adam Mickiewicz University in Poznan.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 73–83
DOI 10.1007/5584_2017_72
© Springer International Publishing AG 2017
Published online: 26 July 2017

Lung Function in Pregnancy in Langerhans Cell Histiocytosis

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Abstract

Pulmonary Langerhans cell histiocytosis (LCH) is a rare disease, affecting usually young people. The course of the disease is variable. In some pulmonary LCH patients a severe lung destruction and progression in spite of chemotherapy is observed, but in others just a cessation of smoking induces a regression of the disease. In the present study we seek to determine the influence of pregnancy on pulmonary function in LCH patients, an unchartered area of research. We addressed the issue by investigating eight pregnant women out of the 45 women hospitalized with the diagnosis of pulmonary LCH in the period from 2000 to 2015. For five of the eight pregnant women it was the second gestation. The median follow-up period was 120 months (range 72–175 months). Ten healthy children were born by a C-section. Two spontaneous miscarriages in the seventh week of gestation, and one tubal ectopic pregnancy were recorded. We found that pregnancy did not significantly influence pulmonary function assessed by the following indices: forced expiratory volume in 1 s (FEV1), lung vital capacity (VC), total lung capacity (TLC), residual volume (RV), diffusing capacity of the lungs for carbon monoxide (DLCO), and the distance and arterial oxygen saturation in 6-min walk test. Only one patient in the third trimester of pregnancy

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experienced bilateral pneumothorax, with persistent air leak. In all patients, delivery and postpartum period were uneventful. We conclude that pregnancy in pulmonary LCH patients is safe and not associated with deterioration of pulmonary function or blood oxygenation.

Keywords
Gestation • Langerhans cell histiocytosis • Pneumothorax • Pregnancy • Pulmonary function • Spirometry

1 Introduction

Langerhans cell histiocytosis (LCH) is a rare disease caused by a clonal proliferation and infiltration of various organs by abnormal bone marrow-derived Langerhans cells. LCH lesions are observed either in a single organ, usually skin, bone, lymph nodes, lungs, or in multiple organs. An unpredictable course of LCH is observed, from spontaneous regression, particularly in adults with pulmonary involvement after smoking cessation, remission with or without relapses, to continuous progression in spite of aggressive chemotherapy (Aricò et al. 2003; Vassallo et al. 2002). Pulmonary LCH is a disease of young and middle aged people, mainly smokers, and it affects equally both genders (Vassallo et al. 2002; Islinger et al. 2000). There are little data on pregnancy in women with pulmonary LCH, and contradictory results have been presented to this end. It has been reported that pregnancy increases the risk of exacerbation of LCH (DiMaggio et al. 1995; Almeida et al. 1984; Ogburn et al. 1981; Morrish and Newhall 1965; Heilbronn and Ridgway 1960). Other reports, however, demonstrate that pregnancy and delivery do not aggravate pulmonary LCH, and both women and the neonates are in good status (Sharma et al. 2006; Tazi et al. 1998; Lavin and Miodovnik 1981). In view of the rarity of pulmonary LCH and the contentious outcomes, the present study seeks to define the influence of pregnancy and delivery on pulmonary function in a series of women suffering from pulmonary LCH.

2 Methods

The study was approved by a local Bioethics Committee of the National Institute of Tuberculosis and Lung Diseases in Warsaw, Poland, and was conducted in accord with the Declaration of Helsinki for Human Research of the World Medical Association.

Forty five women suffering from pulmonary LCH, aged 15–69, were retrospectively evaluated. The valuation covered the period 2000–2015, during which eight women became pregnant, five of them were pregnant twice. The age of women at the time of the first pregnancy ranged from 24 to 36 years and the follow-up period from 72 to 150 months. Basic clinical characteristics and imaging findings concerning the eight patients were presented in Table 1 and pulmonary function results in Figs. 1, 2, 3, 4, 5 and 6. In five of the patients, pulmonary function tests were performed both before and after pregnancy. In addition, in one patient who was diagnosed with pulmonary LCH during pregnancy, and in another who was missed from the observation, pulmonary tests were only available from the time after pregnancy and before pregnancy, respectively. The tests were performed according to the joint guidelines of the American Thoracic Society and European Respiratory Society. The lung volumes were measured with body plethysmography (Jeager MasterScreen; Wuerzburg, Germany) and diffusing lung capacity for carbon monoxide was evaluated with the single breath technique (Standardized Lung Function Testing 1993). The six minute walk

Table 1 Clinical characteristics of patients with pulmonary Langerhans cell histiocytosis (LCH)

Patient	1	2	3	4	5	6	7	8
Lung involvement	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
Diabetes insipidus	Yes	No	Yes	No	No	No	Yes	No
Age at LCH diagnosis (year)	27	29	19	24	26	31	18	23
Time lapse between first symptoms and diagnosis (month)	10	24	6	1	1	6	12	3
Smoking cigarettes (year)	2	7	1.5	2	2	7	2	8
Pneumothorax before diagnosis (n)	6	3	2	1	0	0	0	0
HRCT- typical cystic and nodular lesions in the lungs	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
CT of facial bones	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
MRI of brain	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Bone scintigraphy	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
Pulmonary arterial pressure (mmHg)	25	38	29	21	24	20	22	21

HRCT high resolution computed tomography, *CT* computed tomography, *MRI* magnetic resonance imaging, *Neg* negative result, i.e., no lesion

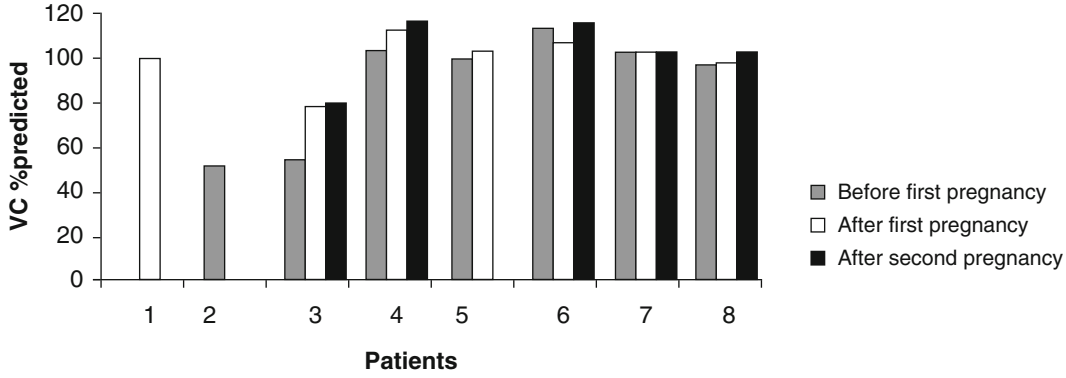

Fig. 1 Vital capacity (VC; % predicted) in patients with pulmonary Langerhans cell histiocytosis (LCH)

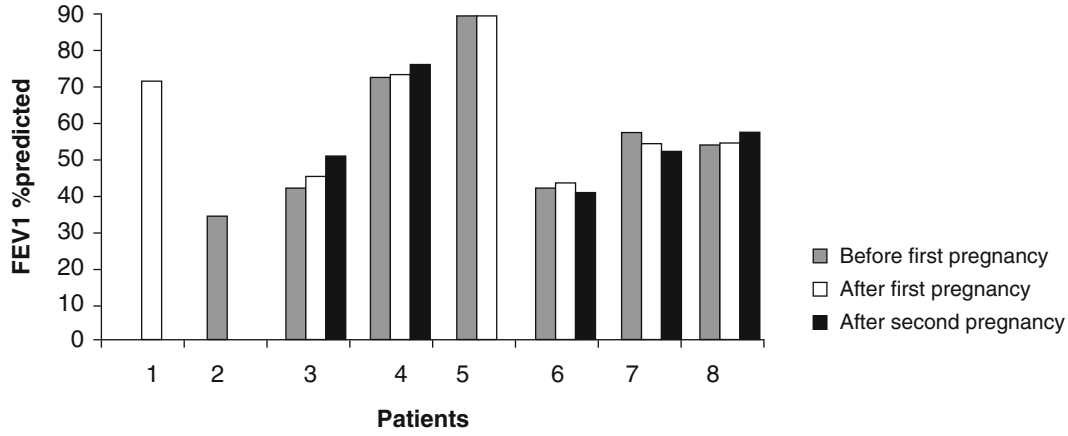

Fig. 2 Forced expiratory volume in 1 second (FEV1; % predicted) in patients with pulmonary Langerhans cell histiocytosis (LCH)

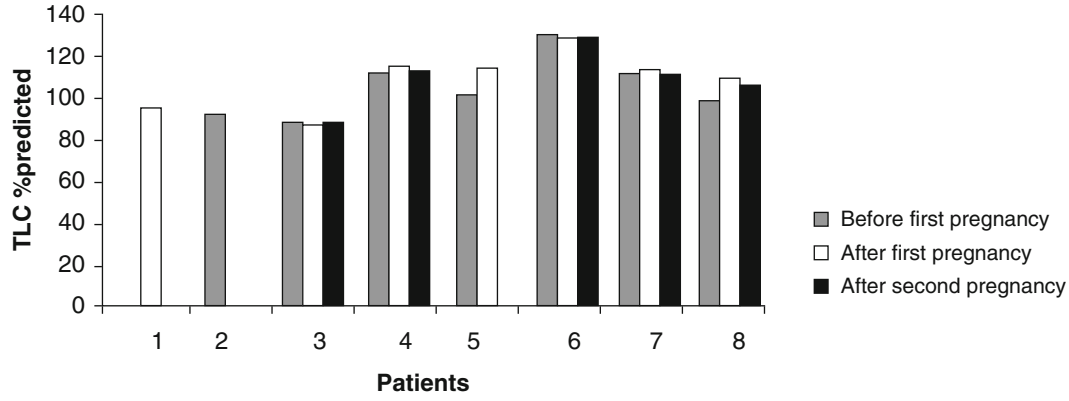

Fig. 3 Total lung capacity (TLC; % predicted) in patients with pulmonary Langerhans cell histiocytosis (LCH)

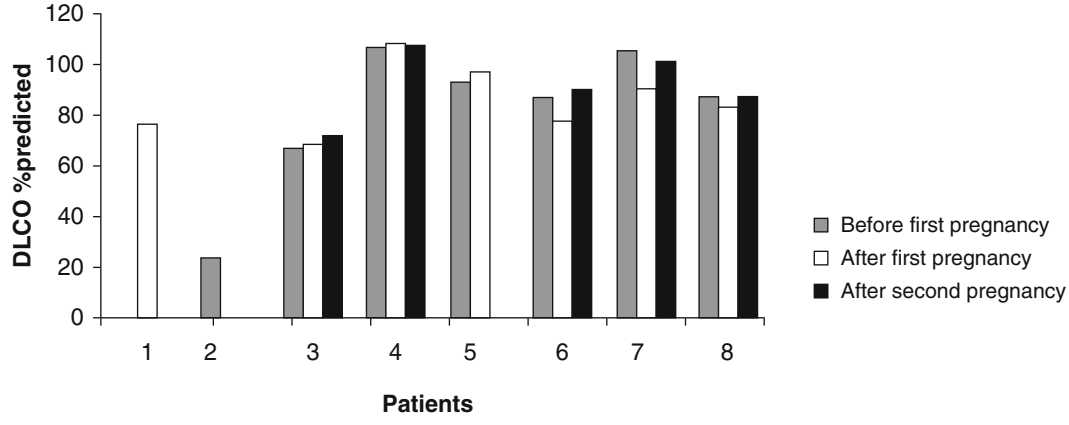

Fig. 4 Diffusion lung capacity for carbon monoxide (DLCO; % predicted) in patients with pulmonary Langerhans cell histiocytosis (LCH)

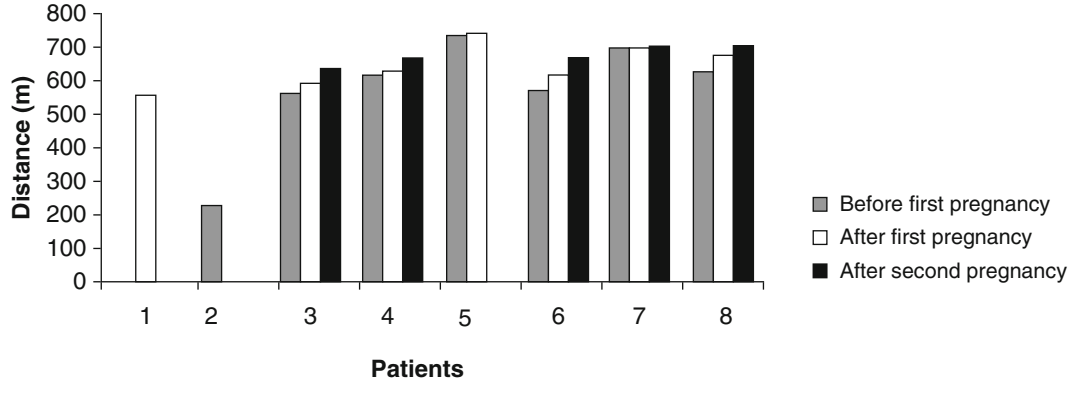

Fig. 5 Distance in six minute walking test (6MWT; m) in patients with pulmonary Langerhans cell histiocytosis (LCH)

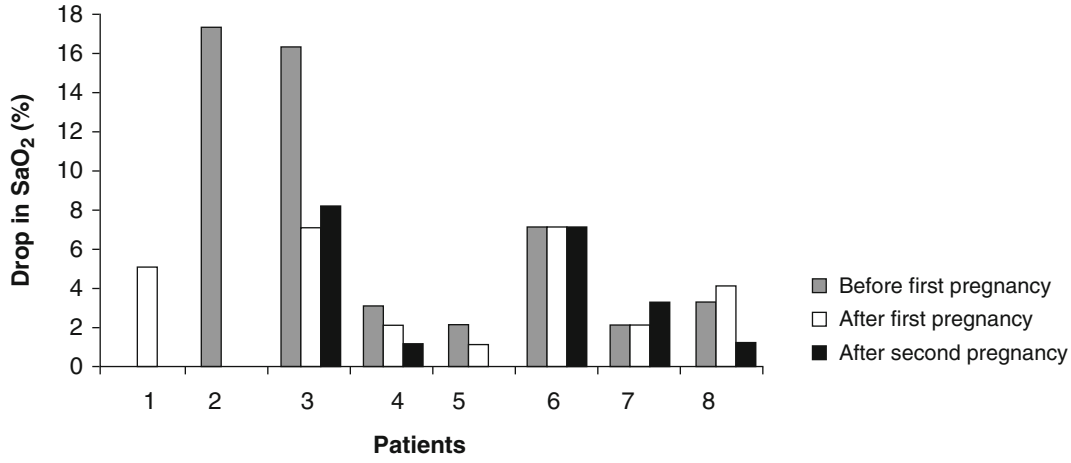

Fig. 6 Desaturation in arterial oxygen content (SaO_2; % of drop from baseline) during in six minute walking test (6MWT) in patients with pulmonary Langerhans cell histiocytosis (LCH)

test (6MWT) was performed according to the ATS recommendations (ATS Statement 2002).

3 Case Reports

3.1 Patient 1

A 27-year-old woman in the fourth month of third pregnancy, who smoked ten cigarettes a day for 4 years and quit 1 year earlier, developed pneumothorax on the right side that was treated with a chest tube drainage. The treatment was ineffective and the patient developed a persistent air leak. After 6 weeks, the right pleurodesis was performed. Subsequently, left pneumothorax occurred, and a chest tube was inserted. At the ninth month of pregnancy she delivered a healthy child through a C-section and then the chest tube was removed. Four months after delivery, the patient was admitted to the hospital with unremarkable symptoms for a health check. Chest computed tomography (CT) disclosed small cystic lesions in both lungs with pleural thickenings. The lesions were reminiscent of lymphangioleiomyomatosis and thus open lung biopsy was performed to confirm the diagnosis. Histological examination of lung specimens revealed proliferation of capillary vessels and the presence of eosinophilic granulomas with CD1a positive cells. The diagnosis of pulmonary LCH and hemangiomatosis were established. The X-ray examination of facial bones, bone scintigraphy, MRI of the pituitary gland and brain, and abdominal ultrasonography all were unremarkable. The patient had been under observation for 8 years, with no progression of the pulmonary lesions. Subsequently, however, the lesions progressed, with a deterioration of lung function and with the appearance of pulmonary hypertension and diabetes insipidus. Chemotherapy was instituted according to LCH Adult 1 protocol, consisting of vinblastine, prednisone, and mercaptopurine for 1 year, and with the additional treatment with desmopressin. The treatment was effective and the patient went into remission that lasted for 5 years. After that, the patient experienced a renewed progression of the disease, for which cladribine treatment was initiated. Stabilization of pulmonary lesions and function was achieved after five therapy courses, which had to be shortened due to neutropenia and pulmonary infections. The patient remained in remission for subsequent 2 years and then died suddenly due to unknown reason.

3.2 Patient 2

A 29-year-old woman (smoker – 8 pack/years), who experienced bilateral pneumothorax for three times, was diagnosed with pulmonary LCH on the basis of histological examination of lung specimens collected at the time of pleurodesis. On admission, she had effort dyspnea, cough, and a weight loss of over 15% of usual body weight. High resolution computed tomography (HRCT) scans showed the presence of multiple cystic lesions with nodules in the lungs and a pleural thickening. Electrocardiography and abdominal ultrasonography, magnetic resonance imaging (MRI) of the brain, CT of facial bones, and bone scintigraphy all were unremarkable. Pulmonary function tests revealed severe bronchial obstruction with lung hyperinflation and with diminished transfer factor for carbon monoxide (Figs. 1, 2, 3, 4, 5 and 6). The patient quit smoking. Nonetheless, deterioration of pulmonary function was observed in the testing performed 6 month later. Prednisone therapy was instituted in a dose of 60 mg *per* day, with some benefit achieved. Subsequent echocardiography examination revealed the presence of pulmonary hypertension. The patient was qualified for lung transplantation. In the meantime, however, during tapering prednisone therapy to 10 mg *per* day, the patient became pregnant. Pregnancy did not result in any further significant deterioration, and a healthy baby was delivered by caesarean section. The patient was missed from the observation after the delivery.

3.3 Patient 3

A 19-year-old woman, (smoker – 5 pack/years), was admitted to the hospital due to the finding of reticulo-nodular lesions in routine chest X-ray examination conducted because of an accident accompanied by a mandible facture. On admission, the patient was in good general condition. Chest CT revealed multiple nodular and cystic lesions in the upper and middle segments of both lungs, with the sparing of costophrenic angles. Ultrasonographic examination of the abdomen, MRI scans of the brain and pituitary gland, and bone scintigraphy were all unremarkable. Cardiac volume parameters and pulmonary arterial pressure were normal. Pulmonary LCH was diagnosed on the basis of histological examinations of lung specimens collected during open biopsy. At the time of diagnosis, polyuria and polydipsia were observed and a dehydration test showed the presence of diabetes insipidus. Chemotherapy according to LCH Adult 1 protocol, with the addition of desmopressin was instituted. Stabilization of pulmonary lesions was achieved with a full course of chemotherapy, and then the patient continued on a low dose of desmopressin. Six months into the chemotherapy, the patient became pregnant. There were no complication during pregnancy and labor. The patient delivered a healthy baby by a C-section at term, weighing 3000 g and having 10 points in the Apgar scale. Two years later, the patient became pregnant again, with uncomplicated delivery of a healthy baby by C-section. The patient remained in remission, with no new pulmonary lesions during a several-year follow-up.

3.4 Patient 4

A 31-year-old woman (smoker – 7 pack/years), with a 3-month long history of flu-like symptoms was admitted to the hospital due to reticulonodular lesions revealed in a routine chest X-ray. Histological examination of specimens collected during video assisted thoracoscopic lung biopsy (VATS) established the diagnosis of pulmonary LCH. The patient's history disclosed that she was born prematurely in the eighth month of pregnancy, and her mother had a blood conflict. The patient had recurrent upper respiratory tract infections, often treated with antibiotics, in childhood. Also, allergy to dust was suspected resulting in the occasional treatment with oral steroids. CT scans revealed typical cysts and nodules, with the sparing of costophrenic angles. Ultrasonographic examinations of the abdomen, thyroid gland and

heart, brain MRI, facial bones CT, and whole body scintigraphy were unremarkable. Cessation of smoking was advised and the patient quit smoking. Subsequently, stabilization of pulmonary lesions and pulmonary function was observed. Three years later, the patient became pregnant; however, she had a spontaneous miscarriage in the seventh week of gestation. One year later, the patient she became pregnant again. Pregnancy and labor were without any adverse events. Medical examinations before and after both pregnancies demonstrated stabilization of radiological lesions and pulmonary function. The patient remained in remission during a several-year follow-up.

3.5 Patient 5

A 22-year-old woman (ex-smoker – 2 pack/years) was diagnosed at the age of 17 with pulmonary LCH on the basis of radiological examination and histological examination of lung specimens collected during VATS. At the time of hospitalization, the patient was in a good general condition. CT scans revealed typical small cysts and nodules, with the sparing of costophrenic angles. Ultrasonographic examinations of the abdomen and heart, CT scans of facial bones, and body scintigraphy were all unremarkable. The patient's pulmonary function results are presented in Figs. 1, 2, 3, 4, 5 and 6. During a 4-year follow-up, pulmonary lesions remained in a stable condition. During that time, the patient became pregnant and delivered a healthy baby through a C-section. Pregnancy and delivery were uneventful. The subsequent control radiological and pulmonary function examinations failed to reveal a progression of the disease.

3.6 Patient 6

A 26-year-old woman (smoker – 2 pack/years) was admitted to the hospital with the diagnosis of pulmonary LCH established on the basis of histological examination of lung specimens collected during VATS. In the medical records, the patient came down with fever and tonsillitis at the age of 17, with *Staphylococcus aureus* identified in blood culture. At that time, antibiotic treatment was administered, with improvement. Six months after the episode, choroiditis was diagnosed. The patient received prednisone at a dose of 1 mg *per* kg orally and depomethylprednisolone in the form of retrobulbar injection, with slight improvement. Tonsillectomy was performed. Chest X-ray examination did not show any abnormalities. One year later, chorioiditis resurged again, with diminished vision in the right eye. The patient was successfully treated and entered remission. However, 4 years later the patient suffered from uveitis, chorioditis, neuroretinitis, and perivasculitis. Prednisone – 40 mg per day was administered for 1 year. All eye lesions were interpreted as being reactive. At that time, a suspicion of an interstitial lung disease was suggested on the basis of X-ray chest examination. Nodular and cystic lesions in both lungs, with the sparing costophrenic angles, and with the hilar enlargement and mediastinal lymph nodes were revealed in chest CT scans. There were no lesions in the brain and facial bones in both CT and MRI scans. Six months after the patient had quitted smoking, deterioration of pulmonary function was observed. Prednisone at a dose of 60 mg *per* day was introduced. Stabilization of pulmonary function was achieved, and the dose of prednisone was tapered. At the time of prednisone dose reduction to 8 mg of methylprednisolone per day, the patient became pregnant for the first time. The dose of methylprednisolone was further reduced to 4 mg *per* day. Pregnancy and labor were uneventful, ending up at term with the delivery of a healthy baby through a C-section. One year later, the patient was pregnant for the second time and also delivered a healthy baby by a C-section, with no medical problems. Stabilization of radiological lesions and pulmonary function was observed (Figs. 1, 2, 3, 4, 5 and 6). The patient was followed-up for 10 years and was in a stable general condition, with slightly improved pulmonary function.

3.7 Patient 7

A 23-year-old women (smoker – 4 pack/years), with bronchial asthma, was admitted to the hospital due to right-sided pneumothorax. Because of the persistent air leak, VATS was performed. Histological examination of lung specimens revealed eosinophilic granulomas, and Langerhans cell displayed CD1a and langerin. CT scans revealed typical cysts and nodules, with the sparing of costophrenic angles. There were no lesions in the brain and facial bones in CT and MRI scans. Echocardiography and abdominal ultrasonography were unremarkable. Cessation of smoking was advised and the patient quit smoking for 3 years, during which time her condition was stable. Later, however, the patient resumed smoking, which caused a deterioration of lung lesions and pulmonary function. In addition, diabetes insipidus and *Mycobacterium xenopi* infection were diagnosed. Antidiuretic hormone replacement therapy and anti-tuberculosis treatment consisting of rifampicin, ofloxacin, ethambutol, and isoniazid were instituted. Two months later, the patient became pregnant for the first time, but she miscarriaged in the seventh week of gestation. Repeat smoking cessation and anti-tuberculosis treatment resulted in improvement of lung lesions and function. The patient became pregnant again 3 years later. This time, pregnancy and delivery by a C-section were without any complications. Chest CT scans showed a stabilization of lung lesions, and pulmonary function did not further deteriorate either, both of which were maintained for a several-year follow-up period.

3.8 Patient 8

A 24-year-old woman (smoker – 10 pack/years) was accidently diagnosed, having no significant symptoms, with reiculonodular lesions in routine chest X-ray examination. Chest CT scans confirmed the presence of small reticulonodular lesions and a few cystic lesions localized mostly in the upper and middle parts of both lungs. Open lung biopsy was performed and pulmonary LCH was diagnosed on the basis of histological examination of lung specimens. At the time of presentation, the patient was in a good general condition. Ultrasonographic examinations of the abdomen, heart, and thyroid gland, brain MRI and facial bones CT scans, and whole body scintigraphy all were unremarkable. After the diagnosis, the patient quit smoking and pulmonary lesions did not progress during a 6-year follow-up period. At the age of 27, the patient became pregnant for the first time. However, tubal pregnancy was diagnosed in the third week of gestation and the right fallopian tube was removed. At the time of extubation after the operation, an episode of bronchial constriction was recorded. Two years later, the patient became pregnant again. Pregnancy and delivery by a C-section were without any complications. Subsequent examinations showed a disease stabilization.

4 Results

The patients clinical features were present in Table 1. All the patients had pulmonary lesions. At the time of diagnosis, one patient had diabetes insipidus, but two others developed it during the follow-up period. In addition, three patients were diagnosed with bronchial asthma, one had obesity and arterial hypertension, and another one suffered from uveitis, chorioditis, neuroretinitis, and perivasculitis. All the patients were ex-smokers, and only one returned to the smoking habit after temporary quitting, which resulted in disease progression. One woman was diagnosed with pulmonary LCH during pregnancy and she experienced bilateral pneumothorax with persistent air leak in the last 4 months of pregnancy.

Patients' pulmonary function was presented in Figs. 1, 2, 3, 4, 5 and 6. Pulmonary function variables remained generally stable during pregnancies. However, in two patients the increases in vital capacity (over 300 ml) and in one patient an increase in forced expiratory volume in one second (over 300 ml) were recorded.

In addition, appreciable increases in 6MWT distance (over 30 m) were observed in patients no. 3, 4, 6, and 8, with less desaturation in patient no. 3 during the test.

5 Discussion

There are no studies and guidelines concerning the procreation in women diagnosed with pulmonary LCH (Girschikofsky et al. 2013). Only a few case reports have been published on the issue. In the present report we review a series of eight patients with pulmonary LCH, who were pregnant at the time of, or became pregnant after, LCH diagnosis. In one woman, pregnancy was connected with persistent air leak that, which dissolved after a C-section. In the remaining patients, pregnancy, and partum and postpartum periods were without any significant adverse events, even though three patients had diabetes insipidus, three had respiratory impairment, and two had pulmonary hypertension. No deterioration of pulmonary function variables was recorded. Likewise, the number of spontaneous miscarriages was akin to that present in the general population (Kutteh 2002).

Clinical course of pulmonary LCH is variable and in the majority of cases smoking cessation induces a stabilization of the disease. Progression of the disease, with respiratory failure, despite aggressive chemotherapy has been described in some cases (Girschikofsky et al. 2013). Five out of the eight patients described in the present report remained stable during pregnancy, and in one patient a slight improvement was noted. The only studies presenting the results of pulmonary function tests during pregnancy in pulmonary LCH patients are those by Lavin and Miodovnik (1981) and Sharma et al. (2006), and the authors have found no appreciable changes in pulmonary function. Cigarette smoking cessation seems the single most important factor influencing the clinical course of pulmonary LCH. In the present study, patients quit smoking. However, a patient who quit smoking but returned to it after a 3-year break experienced a deterioration of pulmonary function and new lesions appeared in the pituitary gland. Active disease, characterized by the reappearance or increase in nodular radiographic abnormalities, increases the likelihood of negative outcomes (Growdon et al. 1986; Tazi et al. 1998). More favorable outcomes have been reported in non-smoking, pregnant LCH patients (Sharma et al. 2006; Lavin and Miodovnik 1981). Nonetheless, DiMaggio et al. (1995) have reported a case of a 27-old-woman with pulmonary LCH and diabetes insipidus, whose condition worsened during pregnancy. The woman delivered a healthy baby by a C-section, but died of LCH progression 18 months later despite intensive chemotherapy. Both pregnancy and LCH progression may exacerbate diabetes insipidus. It has been suggested that vasopressin degradation by placental vasopressinase might be responsible for a worsening of diabetes insipidus during pregnancy (Ananthakrishnan 2009). One of the present patients, who became pregnant after completing chemotherapy, had diabetes insipidus. She was in a good general condition, which enabled the dose reduction of desmopressin after chemotherapy. Likewise, our other patients with diabetes insipidus did not experience a deterioration during pregnancy. In a case of pulmonary LCH reported by Lavin and Miodovnik (1981) dyspnea appeared in the late stage of pregnancy, which was accompanied by a falling level of estradiol. Gestation was prematurely terminated in the thirty sixth week by a C-section, and both the mother and baby were in a good condition. Growdon et al. (1986) have reported a case of histocytosis X in a young woman, a smoker, with a progression from a solitary bone lesion to multiple lesions, among others involving the lungs, skin, vulva, vagina, and cervix. The lesions appeared after a 7-year remission and were apparently related to pregnancies.

Chemotherapy administered in pregnant women diagnosed with LCH seems to have been safe concerning the plausible untoward biological effects on the fetus. However, the evidence on chemotherapy safety is rather indirect and meager. Aviles and Neri (2001) have reviewed the short-term and long-term, reaching a dozen of years or so, side effects in 84 children

born to mothers who received chemotherapy during pregnancy due to hematological malignancies. No ill effects of chemotherapy could be substantiated in the analysis of a host of clinical, psychological, and learning abilities, including the second-generation children. Likewise, Mueller et al. (2009) and Czeizel and Rockenbauer (1997) have failed to demonstrate ill pregnancy outcomes in female survivors of childhood cancer. In general, children of women with LCH, delivered during the active illness, manifest no health abnormalities (Heilbronn and Ridgway 1960). In opposition to the above outlined, Mitra et al. (1994) have reported a case of a female patient with pulmonary LCH whose pregnancy was complicated by fetal growth retardation and oligohydramnios, although it ended up by the delivery of a healthy infant. In the present study, all the children were delivered without any problems, and they had no neurological, psychological, or otherwise health defects. We recorded one spontaneous miscarriage in the seventh week of gestation and one tubal pregnancy in untreated patients with non-active disease, which does not exceed the average of such episodes in the general healthy population. Another miscarriage, also in the seventh week of gestation, was in a patient who was on antituberculosis therapy that by itself could provoke the miscarriage as it is known to have a potential teratogenic effect on the fetus.

There are no as yet guidelines for care of pregnant women with pulmonary LCH (Tschopp et al. 2015; Girschikofsky et al. 2013, Aricò et al. 2003). On average, pregnancy does seem to involve any specific disorders in fetuses or infants delivered by these women. Nonetheless, national registries of LCH patients ought to be created to set the appropriate guidelines in the future.

Acknowledgments Supported by intramural grant no. 7.10 of the National Institute of Tuberculosis and Lung Diseases in Warsaw, Poland.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 85–91
DOI 10.1007/5584_2017_52
© Springer International Publishing Switzerland 2017
Published online: 25 June 2017

Psychobehavioral Effects of Meditation

Mieczyslaw Pokorski and Anna Suchorzynska

Abstract

Meditation is an increasingly popular psychobehavioral therapy. Various meditation techniques in use make it hard to objectively scrutinize the psychological benefits. Therefore, in this study we set out to examine the effects of two fundamentally different meditative techniques, Zazen, 'seated meditation', in which the body and mind are calmed, and Tai Chi, 'meditation in motion', based on energetic martial art performance. The aim was to compare the effects of both techniques on personality structure, emotional intelligence, mood, and coping with stress. The study was conducted in 48 healthy volunteers, aged 39–50, divided into those practicing Zazen, Tai Chi, and the non-meditating controls, each group consisting of 16 persons. The psychometric tools consisted of Coping Inventory for Stressful Situations (CISS), the University of Wales Institute of Science and Technology Mood Adjective Checklist (UMACL), Emotional Intelligence Inventory (INTE), and the NEO Five-Factor Inventory (NEO-FFI). We found that both Zazen and Tai Chi meditations significantly enhanced openness to experience, one of the personality dimensions of the Big Five Model. The enhanced openness was associated with improved strategies for coping with stress. The meditators had less avoidance-oriented approaches to perceived stress. They also had improved mood compared with non-meditating controls. The findings suggest that enhanced openness to experience could shape one's desire to hold onto the meditation regimen. We conclude that both, diametrically different types of meditation, are conducive to mental health by improving the general well-being, counteracting stress, and leading to a better vigor of spirit. Meditation may thus be considered a complimentary, albeit rather modestly acting, adjunct to psychotherapy.

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Keywords
Coping with stress • Meditation • Mental health • Mood • Openness to experience • Personality • Psychotherapy

1 Introduction

Meditation is thought to have a constructive activity concerning psychosomatic health and thus is an attractive form of rehabilitative and anti-stress strategies (Goyal et al. 2014; Shapiro et al. 2005). There are various meditation techniques, with mindfulness meditation and moving meditation situated at the opposing ends of the spectrum. Mindfulness meditation is stationary and is usually practiced in a sitting position, with the practitioners concentrating on a single point of reference such as breathing movements, bodily sensations, or the mantra phrase. Moving meditation, on the other side, or meditation-in-motion is more physical as the practitioners perform a self-paced, series of movements in methodological postures and breathing routines. A common denominator of various techniques is the alleviation of harmful effects of a bustling lifestyle by moving away distracting thoughts, focusing attention on the present moment, which tranquilizes body and mind.

Meditation, being easy to perform and devoid of pharmacological medications, remains an attractive form of behavioral psychotherapy. In particular, building adeptness in managing stressful situations is often seen as the main gain from practicing meditation. The beneficial effects of meditation on the general health are worthy of further exploration. However, little is known of whether the influence of meditation has to do with a type of meditation technique. The present study seeks to define the psychological effects of two fundamentally different meditative techniques, Zazen, 'seated meditation', a spiritual exercise held in Zen Buddhism, whose practitioners calm the body and mind in the sitting lotus-like posture and Tai Chi, Chinese martialart, that advocates soft, but energetic, marital art performance, and thus may be considered 'meditation in motion'. Both meditation techniques, although different in physical expression include deep relaxing breathing. We compared the effects of both techniques on personality structure, emotional intelligence, mood, and coping with stress. We also addressed the issue of the person's psychological attitude that facilitates the undertaking of a meditation course. Perseveration in meditation, as with other stress management routines, requires the determination to stay and succeed in it, otherwise it is bound to fail. The meditation-oriented mindset may thus be accompanied by a specific personality pattern.

2 Methods

The study was approved by the institutional Review Board for Research of the Institute of Psychology of Opole University in Poland. It was based on a self-reported questionnaire survey. A total of 48 healthy volunteers were studied, divided into 3 groups: practicing Zazen, practicing Tai Chi, and the non-meditating control group. The groups were of 16 persons each, with about equal participation of men and women, and the age of the participants ranged from 39 to 50 years. There were some differences in the routine and length of doing meditations between the two groups. The Zazen practitioners were doing the meditation from 1 to 20 years, while Tai Chi was practiced from 3 months to 5 years, with two persons doing it for up to 10 years. Zazen required longer sessions and was often performed in the home setting, while Tai Chi was practiced in outside groups.

The following questionnaires were used: Coping Inventory for Stressful Situations (CISS), the University of Wales Institute of Science and

Technology Mood Adjective Checklist (UMACL), Emotional Intelligence Inventory (INTE), and the NEO Five-Factor Inventory (NEO-FFI).

The CISS inventory consists of 48 items concerning one's style of behavior and actions undertaken in stressful situations. Three styles of coping styles are considered: task-oriented, emotion-oriented, and avoidance-oriented coping (Endler and Parker 1999). The questionnaire helps understand the relationship between the individual's coping style and his or her personality. Responses are scored on a 5-point Likert-type scale. A higher score indicates a greater use of a given coping style. Internal reliability (Cronbach's alpha) of the CISS is high, ranging from 0.72 to 0.92 (Cook and Heppner 1997).

The UMACL assesses the following three dimensions: energetic arousal, tense arousal, and hedonic tone (Matthews et al. 1990). Subjects are asked to rate the applicability of each of the 29 adjectives given in relation to their current mood on a 4-level scale: 'definitely', 'slightly', 'slightly not', or 'definitely not'. The test assesses mood as an affective experience lasting for at least several minutes. The scale has a strong test-retest stability, which makes it little sensitive to the possible influence of personality traits, particularly extraversion/neuroticism, or mood. It may thus be assumed that the UMACL is a measure that reflects the state rather than trait.

The INTE inventory, developed by Schutte et al. (1998) and adapted for the Polish population by Ciechanowicz et al. (2000), was used to assess the emotional intelligence, defined as the ability to understand and control one's emotions and actions and to use that knowledge to manage someone else's actions. The measure consists of 33 items scored on a 5-point Likert-type scale and has a high internal consistency (Cronbach's alpha) ranging from 0.83 to 0.87.

The NEO-FFI assesses the relations among the five factor model of personality traits defined as follows: (1) openness to experience – curiosity, independent judgment; (2) conscientiousness – self-control in planning and organization, (3) extraversion – active sociability, positive emotionality; (4) agreeableness – altruism, empathy, cooperative skills; and (5) neuroticism – negative emotions and distress rising in response to stressful situations. The inventory consists of descriptive statements rated on a 5-point Likert- type scale. Each of the five personality domains consist of 12 items, giving the total score of 60 points, and each can be scored independently. Domains have a high internal consistency, with Cronbach's alpha ranging from 0.68 to 0.86 (Costa and McCrae 1992).

Data were given as means ±SD of raw scores. The statistical differences among the three study groups were evaluated with one-way analysis of variance ANOVA. Significant differences were defined as a p-value <0.05. A commercial statistical package of Statistica v14 (StatSoft; Tulsa, OK) was used for all comparisons.

3 Results

We found that Zazen meditation appeared the strongest modifier of personality structure. The disciples of both Zazen and Tai Chi had a significantly higher level of openness to experience than the non-meditators. However, Zazen practitioners displayed appreciably less extraversion and conscientiousness than both Tai Chi and non-meditating individuals ($p < 0.05$). These last two personality dimensions were in Tai Chi practitioners at a level comparable to that in the non-meditators ($p < 0.05$) (Fig. 1).

We further found that the task-oriented style of coping with stress predominated in all three groups of subjects studied: non-meditating, and Zazen and Tai Chi meditating. There were no appreciable differences among the three groups concerning the task-oriented and emotion-oriented styles. However, individuals practicing both Zazen and Tai Chi significantly less engaged into a disadvantageous, avoidance-oriented style of coping with stress than the non-mediating ones ($p < 0.05$) (Fig. 2).

Concerning the mood dimensions, hedonic tone predominated in all three groups of subjects studied, closely followed by a high level of energetic arousal, with no appreciable differences

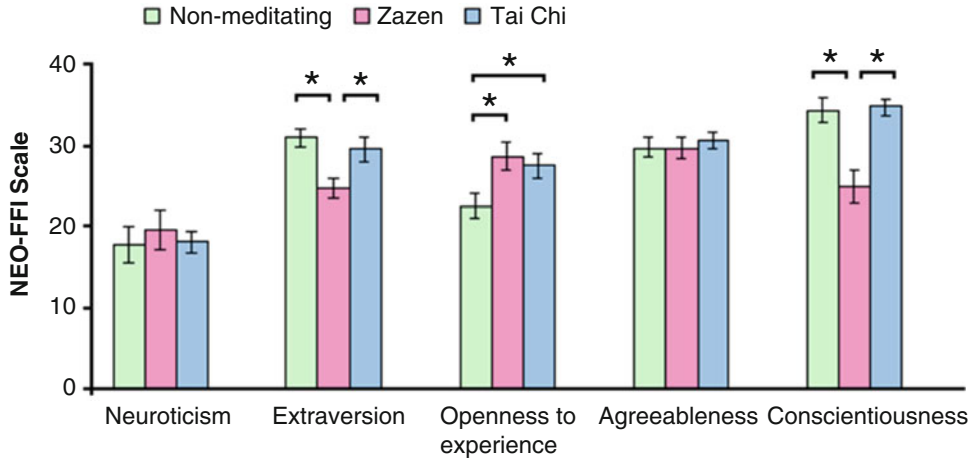

Fig. 1 Personality structure in meditating and non-meditating individuals (*NEO Five-Factor Inventory – NEO FFI*). Data are means ±SD of raw scores; *p < 0.05

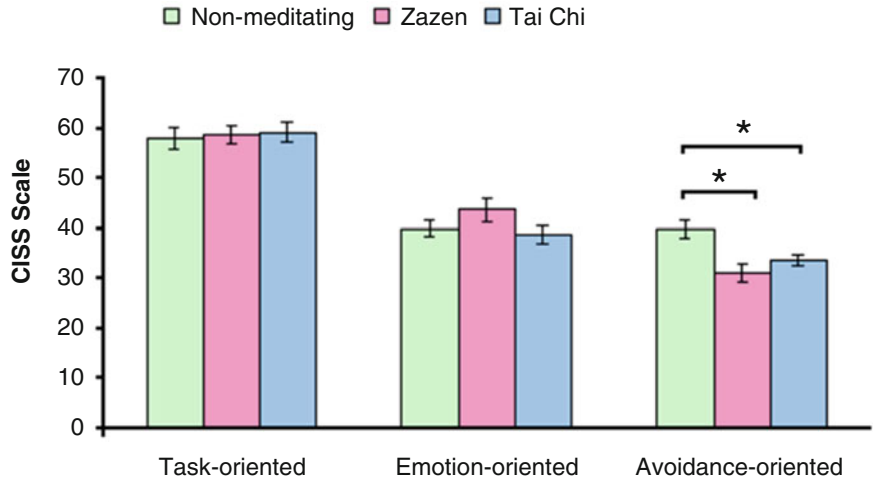

Fig. 2 Coping with stress (*Coping Inventory for Stressful Situations – CISS*). Data are means ±SD of raw scores; *p < 0.05 for the differences between Zazen and Tai Chi practitioners *versus* non-meditating controls

among the three groups. The level of tense arousal was significantly lower in all groups; it was about halved compared with both hedonic tone and energetic arousal. However, it was further decreased in both meditations, with a significant decrease in those practicing Zazen compared with non-meditators ($p < 0.05$) (Fig. 3).

Emotional intelligence was not different between the two types of meditation techniques. Nor was it different from that present in the non-meditating controls (Table 1).

4 Discussion

An inspiration for this study was the increasing popularity of meditation in the area of behavioral psychotherapy aimed at relaxation and anti-stress effects. Meditation produces changes in both body and mind. There is evidence that meditation exerts a positive influence on somatic health, for instance, enhancing the immune brain-function and reducing the pro-inflammatory gene expression or modulating leukocyte function related to

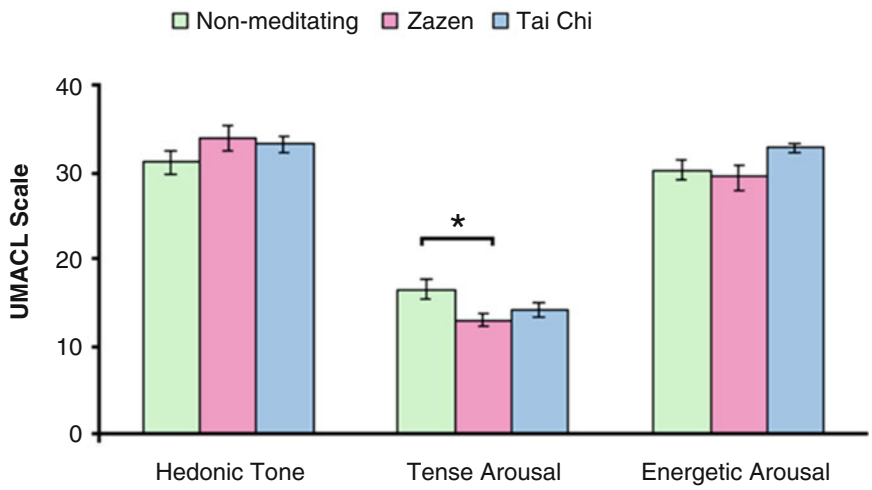

Fig. 3 Mood assessment (*University of Wales Institute of Science and Technology Mood Adjective Checklist – UMACL*). Data are means ±SD of raw scores; *p < 0.05 for the differences in tense arousal between Zazen practitioners and non-meditating controls

Table 1 Level of emotional intelligence in the meditating and non-meditating individuals (*Emotional Intelligence Inventory – INTE*)

Non-meditating	123.3 ± 12.6
Zazen	121.5 ± 14.3
Tai Chi	128.1 ± 13.5

Data are means ± SD of raw scores

inflammatory responses (Muehsam et al. 2017; Bower and Irwin 2016); clear aspects of the mind-body connection. Purely psychological benefits of meditation are less certain. In the psycho-sphere, meditation can hardly be considered evidence-based behavioral therapy. The issue is confounded by a range of variations in meditative techniques in use; often not standardized and scientifically scrutinized. The present study addressed this issue of variable meditation by examining the effects on basic psychological dimensions, such as the personality traits, the ability of coping with stress, mood, and the emotional intelligence, of two distinctly different meditation techniques: Zazen derived from Zen Buddhism's spiritual exercise in the seated posture and Tai Chi derived from energetic motion of Chinese marital art. These meditation techniques are philosophically at extreme ends that can be called 'calmed calm' and 'excited calm', but both techniques are considered to help control the self, keep emotions at bay, remove ego, and stay in the moment – 'staying within yourself'. The present study unraveled a significant, albeit modest, influence of both Zazen and Tai Chi practice on personality structure, consisting mainly of increased openness to experience; one of the dimensions of the Big Five Model of personality of McCrae and John (1992). Openness entails attentiveness to one's feelings and values, imagination, creativity, and tolerance for unorthodox ideas and behaviors. It seems to be a positive personality trait in that it helps maintain a subjective feeling of well-being (Steel et al. 2008). Openness is associated with the interest in acquiring new knowledge and learning, and the engagement in intellectual and sensory diversity, as opposed to closed people who are characterize by mental conservatism and have the preference for experiences they are familiar with (McCrae 1987). All that makes openness to experience positively associated with the general feeling of well-being, intelligence, and also with positive mood, and inversely associated with avoidance-oriented strategy (Ihle et al. 2016; Steel et al. 2008). The present findings confirm the association of openness for experience with positive anti-stress effects. Both Zazen and Thai Chi practitioners, in comparison with

non-meditators, displayed a significantly lower inclination to use the avoidance-oriented manner in stressful situations, with an apparent trend toward more hedonistic attitude in the state of mind and mood. However, the lack of an evident increase in the style concentrated on task falls short of the intuitive expectations from meditation. Thus, we demonstrate that meditators not so much improve their way of coping with stress, as that non-meditators fare worse due to the more frequent use of avoidance in straining situation. Nonetheless, the fact remains that meditators are able to fare better in stress. Favorable effects of mind training on quality of life, mood, and perceived stress have been reported in previous studies (Wang et al. 2016; Lane et al. 2007; Davidson et al. 1976; Goleman 1976).

The present study cannot resolve the neurophysiological background of enhanced openness to experience in response to meditation. The clue may lie in the consciousness-affecting effects of meditation. In a study by Kasamatsu and Tomio (1966) changes in the EEG activity were recorded during Zazen meditation, concerning mostly the alpha waves that decrease with time of meditation. The alpha blocking (dehabituation) to the repeated click stimuli observed in that study points to the possibility of meditation-induced hypnogenic influence. Openness to experience may associate with a hypnogenic disposition of attention for the engagement in self-absorbing resources, which alters the reception of reality (Tellegen and Atkinson 1974).

In the present study, the enhanced openness for experience was a highlighting feature of personality structure during both Zazen and Tai Chi meditations. Openness mirrors a person's general curiosity about things and people, and a desire to come to know the inner self better. Openness could also drive the undaunted perseverance in meditation despite the effort and time it usually takes. In Zazen, however, openness was associated with decreases in conscientiousness and extraversion, both having a negative connotation. These decreases were absent in Tai Chi. Lower conscientiousness, in particular, may reflect less care and attention in carrying out tasks. Zazen meditation is a technique that is practiced alone in an introverted and indrawn manner as opposed to more sociable group practicing of Tai Chi, which may distort otherwise positive effects on personality of Zazen. Less conscientiousness, often characterized by low motivation for social achievements, seems in line with lower extraversion that, in turn, is associated with the preference for staying alone. That may be a reason for choosing to meditate alone at home rather than during group meetings.

The present findings failed to show differences in the level of neuroticism during either meditation. That is at variance with the starting premises of the study stemming from the works of others (Telles et al. 2012; Sandahl 1980; Williams et al. 1976) who show deceased neuroticism in meditating persons. We also failed to show that meditation could have an enhancing effect on emotional intelligence and related to it empathy or the ability to discern and control emotions. The reasons for these discrepancies are not readily apparent. Plausibly, they may lie in psychometric tools and methodological approaches used as well as in the population samples that widely differ in this area of psychological studies.

In conclusion, the present study demonstrates that both Zazen and Tai Chi, diametrically different meditation techniques in terms of motion and energy, changed the personality structure leading to a significant increase in openness to experience, compared with the non-meditating persons. The increased openness was associated with improved strategies of coping with stress. The meditators had less avoidance-oriented attitude to perceived stress; the style with a negative connotation for anti-stress strategy. They also had improved mood. The findings suggest that enhanced openness to experience could be influential in holding onto the meditation regimen. Practicing Tai Chi may have an edge over Zazen in psychobehavioral gain, as the latter decreases extraversion and conscientiousness; the qualities that characterize the lonely and introverted technique of Zazen meditation and that may be considered undesirable. Overall, both types of meditation are conducive to mental

health by improving the general well-being, counteracting stress, and leading to a better vigor of mind and spirit. Meditation may thus be considered a complimentary, albeit rather modestly acting, adjunct to psychotherapy.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 93–100
DOI 10.1007/5584_2017_69
© Springer International Publishing AG 2017
Published online: 21 July 2017

Vaccine Effectiveness against Influenza in 2015/16 in Hospital and Ambulatory Medical Care Facilities: Polish Results of the European *I-MOVE+* Multicenter Study

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Abstract

Influenza vaccination is the best measure available to prevent seasonal influenza infection. The majority of studies on vaccine effectiveness in the 2015/16 season conducted in the European *I-MOVE+* Project, show that a match between the circulating influenza strains in the general public and those included in the vaccine for the Northern Hemisphere was low to moderate. As part of *I-MOVE+*, Poland has implemented a case control negative study design and molecular biology methods, such as real time RT-PCR, to assess the vaccine match and effectiveness. The research described herein consisted of two major influenza vaccine effectiveness investigations conducted in Poland in the 2015/16 season. The general practice part of the study included 228 cases consisting of 159 type A, 65 type B, and 4 coinfections (types A + B), and 312 negative control cases. The hospital study part included 26 cases consisting of 21 type A, 2 type B, and 3 coinfections, and 13 negative control cases. The data were collected from patients of all ages recruited by 46 volunteering doctors in 15 Poland's provinces and three hospitals, respectively. In both study parts, only were seven patients and 12 control subjects vaccinated. Low vaccine coverage, a major limitation of the Polish study, makes the calculation of vaccine effectiveness for the Polish population hardly applicable statistically. Despite the crudeness of data, they were included

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into the common European analysis. The overall vaccine effectiveness amounted to 21.0% (95% CI: 74–122). It was somehow better for type B virus: 53.9% (95% CI: 47–87) and type A virus: 23.6% (95% CI: 83–185). A larger sample size is needed to achieve a desired interpretation of results on influenza vaccine effectiveness in Poland.

Keywords
Age • General population • Influenza • Vaccination • Vaccine coverage rate • Vaccine effectiveness

1 Introduction

Influenza is an infectious disease with an important public health impact at the global level. Annual epidemics of seasonal influenza represent a significant burden on society. The emergence and dominance of circulating influenza viruses are variable and difficult to predict. Therefore, influenza surveillance plays an important role in disease prevention (Blank et al. 2012; Council of the European Communities 2009). Studies on influenza vaccine effectiveness coordinated by the EpiConcept (I-Move in Europe 2016; Kissling and Valenciano 2016) and ECDC (2016) have been conducted in the EU since 2007. The main goals of those studies have been to assess a match between the circulating viral strains and those included in the vaccine as well as vaccine effectiveness in the general public. Poland has joined these studies as of the 2010/11 epidemic season. Recently, the country has implemented a case control negative study design and molecular biology methods within the European *I-MOVE+* Multicenter project on influenza vaccine effectiveness.

In the 2015/16 influenza season in Europe, circulation of influenza type A viruses predominated over type B. The A(H1N1)pdm09 virus was found in the majority of infected patients in intensive care units. Studies on influenza vaccine effectiveness conducted in the 2015/16 season in the European *I-MOVE+* project have shown that a match between the circulating vaccine strains and those included in the vaccine for the Northern Hemisphere was low to moderate. The goal of this report is to present the findings of the Polish part of the *I-MOVE+* study on influenza vaccine effectiveness in the 2015/16 epidemic season.

2 Methods

This study was approved by a local Ethics Committee of the National Influenza Center, National Institute of Public Health- National Institute of Hygiene in Warsaw, Poland and was conducted in accord with the principles of the Declaration of Helsinki for Human Research. The adult participants and legal guardians in case of children gave informed written consent to be included in the study. All participants were interviewed using a standardized questionnaire to collect data on clinical, epidemiological, and descriptive information, including the date of illness onset, the date of swabbing, the vaccination status, and the presence of any chronic health conditions. The study on the influenza vaccine effectiveness conducted in the 2015/16 season in Poland consisted of two major parts; the general practice part and the hospital part. The former was based on the collaboration with 46 volunteering general practitioners (GP) from 28 ambulatory medical care facilities from across the country, representing 15 Voivodship Sanitary Epidemiological Stations. There were 540 patients in this study part, 226 men and 314 women. Each GP recruited at least 5 patients selected for the study. The later part was conducted in three regional hospitals; two in

central and one in southern Poland and included 40 patients; 15 men and 25 women.

Nasopharyngeal swab specimens were collected from persons suffering from influenza-like illness (ILI) or acute respiratory illness. The specimens were tested for influenza viruses by real-time reverse transcription polymerase chain reaction (RT-PCR). Detection of a specific viral nucleic acid was considered a positive influenza diagnosis. The control group consisted of patients who did not meet the definition of ILI and had negative influenza RT-PCR results. All specimens were transported to the laboratory with the attached information concerning the date of general practitioner's visit and the date of swabbing. The participants of both ILI and non-influenza illness were stratified by age to the following groups: 0–14, 15–64, and 65+ years old.

2.1 Laboratory Elaboration

Laboratory elaboration consisting of virus detection, typing, and subtyping was conducted in the Department of Influenza Research of the National Influenza Center in the National Institute of Public Health – National Institute of Hygiene in Warsaw, Poland, and in Voivodship Sanitary Epidemiological Stations.

Influenza virus RNA was isolated using a Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega Corporation; Madison, WI) from 200 μl of clinical samples suspended in phosphate-buffered saline, according to the manufacturer's instructions for low elution volume cartridges. The RNA was eluted with 50 μl of RNAse-free water.

Real-time RT-PCR was performed with a Light Thermocycler 2.0 System (Roche Diagnostics; Rotkreuz, Switzerland) according the method described previously (Hallmann-Szelińska et al. 2016). Briefly, reactions were conducted in capillary tubes of 20 μl volume using 0.5 μl (20 nM) of primers and 0.5 μl (5 nM) of probes for each reaction. The reaction mixture consisted of $MgSO_4$, bovine serum albumin (BSA), RNase-free H_2O, and SuperScript® III/Platinum® *Taq* Mix (Invitrogen by Life Technologies – Thermo Fisher Scientific, Carlsband, CA), and was incubated with 5 μl of RNA sample in each capillary tube. RNA from the 2015/16 vaccine viruses: A/California/7/2009(H1N1)pdm09 and A/Texas/50/2012 (H3N2), and B/Massachusetts/2/2012 were introduced as positive controls. The negative control constituted RNase-free water. Before DNA amplification, RNA templates were reverse transcribed (at 50 °C for 30 min) to obtain the corresponding cDNA. Subsequently, cDNA was subjected to denaturation (one cycle at 95 °C for 2 min), followed by further steps of denaturation (95 °C; 15 s), annealing (55 °C; 30 s), and extension (72 °C; 20 s) repeated in 45 cycles.

2.2 Statistical Elaboration

Influenza vaccine effectiveness (VE), a single percentage value estimating the reduction in risk provided by the vaccine in laboratory-confirmed ILI, was calculated as 1 – OR; where OR is the odds ratio for acquiring influenza infection in vaccinated *versus* unvaccinated patients with laboratory confirmed ILI. Logistic regression analysis was used to calculate the adjusted OR and its correspondent 95% confidence interval (CI). The statistical analysis was performed with a commercial Stata 12 package (StataCorp LLC; College Station, TX).

3 Results

In the general practice part of the study, there were 228 influenza positive and 312 negative control cases. The positive cases consisted of 159 influenza type A, including 136 A/H1N1/pdm09 and 23 type A unsubtyped, 65 type B, and four type A + B co-infections. The second hospital part included 26 cases consisting of 21 type A, 3 type B, and 2 co-infections, and 14 control cases.

Among all 540 patients investigated, there were 35 persons aged 65+, with positive influenza results in 12 of them. These positive results

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Table 1 Positive influenza type A and B cases and negative control cases in the *I-MOVE+* Polish study among ambulatory medical care facilities during the 2015/16 epidemic season

		Positive influenza cases (n = 228)	Negative control cases (n = 312)
		n (%)	n (%)
Age-groups (year)	0–4	19 (8.3)	38 (12.2)
	5–14	35 (15.4)	35 (11.2)
	15–64	162 (71.1)	216 (69.2)
	65+	12 (5.3)	23 (7.4)
Gender	Male	94 (41.2)	132 (42.3)
	Female	134 (58.8)	180 (57.7)
Vaccination	Yes	7 (3.1)	12 (3.8)
	No	221 (96.9)	300 (96.2)
Influenza type	A/H1N1/pdm09	136 (59.6)	–
	A/H3N2/	0 (0)	–
	A unsubtyped	23 (10.1)	–
	B/A co-infection	1 (0.4)	–
	B Yam/A co-infection	0 (0)	–
	B Vic/A co-infection	3 (1.3)	–
	B Yamagata	0 (0)	–
	B Victoria	9 (3.9)	–
	B unknown	56 (24.6)	–
Any chronic condition, including obesity and pregnancy	Yes	42 (18.4)	62 (19.9)
	No	186 (81.6)	250 (80.1)
Belongs to target group for vaccination	Yes	121 (53.1)	177 (56.7)
	No	107 (46.9)	135 (43.3)

were typed/subtyped as follows: nine cases of A/H1N1/pdm09, one case of influenza type A unsubtyped, and two cases of influenza type B. A descriptive summary of *I-MOVE+* findings from both GP ambulatory and hospital medical care facilities is presented in Tables 1 and 2, respectively.

Virological characteristics of weekly specimens during the 2015/16 season showed that influenza type A virus predominated in the circulation. The collection of samples started in Week 2 (W2), according to the International Organization for Standardization (ISO) week-numbering. The seasonal peak took place in W6, confirming a typical course of the epidemic curve (Fig. 1). In the hospital part of the study, severe acute respiratory infections (SARI) cases predominated in W8 and W9, still providing a significant number of influenza cases down to W14 (Fig. 2).

4 Discussion

The 2015/16 influenza season in Europe was characterized by a high degree of antigenic and genetic mismatch between the circulating type A viruses and the vaccine strains consisting of A/California/7/2009(H1N1)pdm09 clade NYMC X-181 and A/Switzerland/9715293/2013 (H3N2) clade NIB-88, recommended for the Northern Hemisphere in the season.

In Europe, there are considerable differences in the state of vaccination against seasonal influenza in populations at risk of a severe and complicated course of influenza. The target 75% coverage of vaccination recommended by the Council of the European Union (2009) is seldom met. The UK and the Netherlands are the only two countries that have reached or nearly reached this target level in the elderly. Vaccination may

Table 2 Positive influenza type A and B cases and negative control cases in the *I-MOVE+* Polish study among ambulatory medical care facilities during the 2015/16 epidemic season

		Positive influenza cases (n = 26) n (%)	Negative control cases (n = 14) n (%)
Age-groups (year)	65–79	16 (61.5)	10 (71.4)
	80+	10 (38.5)	4 (28.6)
Gender	Male	7 (26.9)	8 (57.1)
	Female	19 (73.1)	6 (42.9)
Vaccination	Yes	1 (3.8)	0 (0)
	No	25 (96.2)	14 (100)
Influenza type	A/H1N1/	16 (61.5)	–
	A/H3N2/	0 (0)	–
	A unsubtyped	5 (19.2)	–
	B Yam + A/H3N2/ co-infection	1 (3.9)	–
	B Vic + A/H3N2/ co-infection	1 (3.9)	–
	B unknown	3 (11.5)	–
Hospital ward	Internal medicine	7 (26.9)	9 (64.3)
	Emergency department	0 (0)	0 (0)
	Intensive care unit	0 (0)	2 (14.3)
	Pulmonary diseases	0 (0)	0 (0)
	Cardiology	16 (61.5)	2 (14.3)
	Infectious diseases	0 (0)	0 (0)
	Geriatrics	0 (0)	0 (0)
	Others	3 (11.6)	1 (7.1)

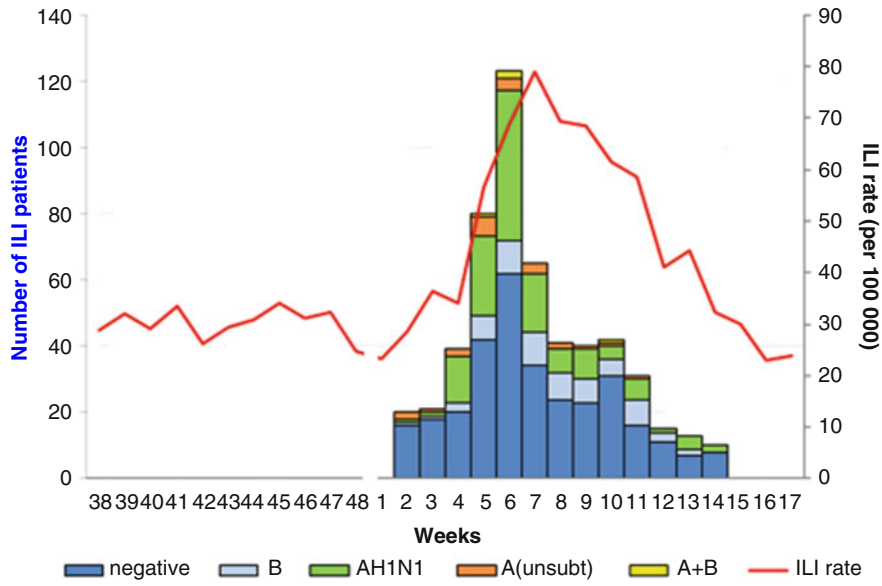

Fig. 1 Virological characteristics of nasopharyngeal swab specimens collected in the general practice facilities within the *I-MOVE+* study distributed by the week number during the 2015/16 influenza epidemic season in Poland; week-numbering is in accordance with the International Organization for Standardization (ISO) system

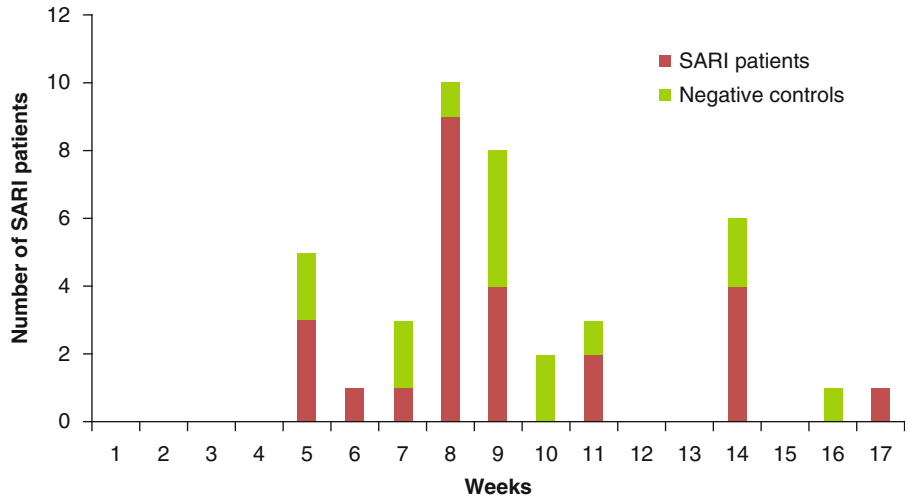

Fig. 2 Severe acute respiratory infections (SARI) in hospitals involved in the *I-MOVE+* study distribution by the week number during the 2015/16 influenza epidemic season in Poland; week-numbering is in accordance with the International Organization for Standardization (ISO) system

reduce the risk of infection by influenza viruses by 70–80% in healthy adults and by 30–70% in the elderly (Centers for Disease Control and Prevention 2013). The implementation of vaccination programs against influenza is cost-effective, taking into account the reduction in morbidity and mortality due to the disease and its complications. It should be noted that vaccine effectiveness may vary depending on the season. The relevance of vaccine strains circulating in the population in previous years does not provide cross-protection (ECDC 2016; Grohskopf et al. 2014; Council of the European Communities 2009). In Poland, influenza vaccine coverage in the 2015/16 epidemic season was dismally low, amounting to barely 3.55% of the population. This low coverage has persisted for years in both general population and subpopulations or age-groups at high risk such as the immunocompromised, children, or pregnant women. A somehow better vaccination coverage, amounting to 6–8%, concerns the elderly, which may stem from the vaccination cost reimbursement currently used in this population group. It thus may be presumed that the Polish population, as a whole, is outstandingly susceptible to influenza infection and complications. The death toll due to influenza infection amounted to 140 cases in the 2015/16 season in Poland. That pointedly demonstrates a high price paid by the society in terms of hospitalizations, work absenteeism, and overall medical care.

Vaccine coverage data are part of the calculation of vaccine effectiveness (VE). A low value of vaccine coverage makes the calculation of VE for the Polish population statistically inapplicable. The overall VE amounted to 21.0% (95% CI: 74–122). It was somehow better for type B virus: 53.9% (95% CI: 47–87) and type A virus: 23.6% (95% CI: 83–185). Despite the crudeness of these data, they were included into the common European analysis. The European analysis has shown that the adjusted VE was low to moderate for A/H1N1/pdm09: overall 33.1% and by the age-groups: 31.7%, 42.5%, and 10.0% among 0–14, 15–64, and 65+ years-old individuals, respectively. The Italian studies show a good VE against A/H1N1/pdm09 and B viruses and the lack of VE against A/H3N2/virus due to the antigenic mismatch between the circulating A/H3N2/and the respective 2014/15 vaccine strain (Rizzo et al. 2016). The I-MOVE Multicenter Case-Control Study has confirmed a moderate VE against A/H1N1/pdm09 and B viruses, and a low VE against A/H3N2/2014/2015 virus in the 2014/15 epidemic season; the latter was

consistent with a reported mismatch between the circulating and vaccine strains (Valenciano et al. 2016). In contrast, Pebody et al. (2016) have reported moderate to good levels of protection, amounting to overall adjusted end-of-season VE of 52.4% in children in the UK. The country was in the third season of introducing universal pediatric influenza vaccination with a quadrivalent live attenuated influenza vaccine. These results may be viewed as a reassurance of the efficacy of modern influenza immunization programs in modern health protection and care.

5 Conclusions

- Studies on vaccine effectiveness are a useful epidemiological and microbiological tool for the assessment of influenza vaccine effectiveness in prevention against infection.
- Influenza vaccine coverage is dismally low, amounting to a few percentage points, in the general population in Poland.
- Overall vaccine effectiveness in the epidemic 2015/16 season in Poland amounted to a low 21.0% (95% CI: 74–122). It was somehow better for type B virus: 53.9% (95% CI: 47–87) and type A virus: 23.6% (95% CI: 83–185).
- Low vaccine coverage, making the calculation of vaccine effectiveness statistically inapplicable, was a major limitation of the Polish study. Nonetheless, the Polish results were included inti the European *I-MOVE+* Multicenter Study.

Acknowledgements We are grateful to the EpiConcept and ECDC for giving an opportunity to carrying out the study in Poland. Supported in part by the I-MOVE+ Project and H2020 funds.

Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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Advs Exp. Medicine, Biology - Neuroscience and Respiration (2017) 35: 101–102
DOI 10.1007/5584_2017
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