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Increased Risk of Lung Metastases in Patients with Giant Cell Bone Tumors: A Systematic Review

Josef Yayan

Abstract

Giant cell tumors of the bone are rare, usually benign, tumors consisting of large, multinucleated bone cells. Remarkably, these tumors are characterized by aggressive growth. They tend to recur frequently and, in rare cases, metastasize to the lungs. Previous studies tried to identify risk factors for lung metastasis by giant cell bone tumors. Those studies reported different results due to a small number of patients. Therefore, a particularly high risk associated with this type of bone tumor prompted this systematic review and meta-analysis to identify risk factors for the development of lung metastases. The risk factors for lung metastasis by giant cell bone tumors searched for in this study were gender, age, lung metastasis and recurrence period, follow-up time, primary or recurrent tumor, Campanacci grading, tumor localization, disease course, treatment of primary and recurrent tumors, and pulmonary metastases treated by surgery, radiation, and chemotherapy. This meta-analysis identified the features outlined above by comparing the groups of patients with giant cell bone tumors and lung metastases with the control group consisting of patients without lung metastases. The search for suitable studies revealed 63 publications with a total of 4,295 patients with giant cell bone tumors. Of these, 247 (5.8%; 95% confidence interval (95%CI) 5.1–6.5%) patients had lung metastases. Further, the risk factors for lung metastases were the following: recurrence ($p < 0.0001$), lung metastasis time ($p < 0.0001$), Campanacci grade II ($p = 0.028$) and grade III ($p = 0.006$), localization in the lower limbs ($p = 0.0007$), curettage ($p = 0.0005$), and local irradiation of the primary tumor ($p = 0.008$). All studies showed a high-risk bias due to the absence of blinding of the participants, personnel, and outcome assessment. Special attention should be paid to tumor recurrence in the long follow-up time, since more advanced giant cell bone tumors, particularly in lower extremities, tend to reoccur and metastasize to the lung. Surgical treatment and local irradiation should be performed thoughtfully, with extended follow-up periods.

Keywords

Giant cell bone tumor · Lower limbs · Lung metastases · Meta-analysis · Osteosarcoma · Risk factors · Tumor recurrence

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1 Introduction

Giant cell tumors of the bone are rare tumors that are found mostly in the epiphysis of long bones. Although they are classified as benign, they often exhibit locally aggressive growth, have a high risk of recurrence, and, in rare cases, metastasize to the lung (Fletcher et al. 2018; Werner 2006). Histologically, they are characterized by monocytes and mesenchymal stromal cells in addition to osteoclastic giant cells (Elder et al. 2007). Ordinarily, such tumors develop in the second and third decade of life (Kundu et al. 2018), and they are commoner in women than in men (Hu et al. 2016). Affected patients present with subacute to acute pain mainly during exercise (Mavrogenis et al. 2017). In addition, patients often present with newly occurring pathological bone fractures (Cao et al. 2017).

Following the initial findings, discovery of a cystic, juxta-articular, nonresponsive mass via radiography often results in a trial biopsy and curative intervention (He et al. 2017). Surgical management is of great importance, since tumors tend to recede due to its biological properties, but aggressive growth of some local lesions endangers joint preservation. For that reason, surgical en bloc resection, followed by curettage and subsequent filling of defects with bone cement, has become an established treatment for the epiphysis of long bones (Pazionis et al. 2013). Furthermore, after local surgical rehabilitation, radiotherapy is applicable to tumor sites that are not easily accessible, where sufficiently radical surgery with satisfactory results is hardly feasible (Shi et al. 2013). Adjuvant chemotherapy should be proposed for giant cell bone tumors of medium- and high-grade malignancy when the tumors progress and become inoperable (Liang 2018).

In principle, the extent to which lung metastases require pulmonary surgery depends on the number and localization of lung metastasis and tumor-free resection margin. Individual lung lesions can be easily removed by complete resection. Less often, lung wedge resection or lobectomy is required. In case of diffuse metastatic lungs or technical or functional inoperability, radiotherapy may be used in addition to local surgical procedures (Klenke et al. 2011).

The aim of this review was to identify risk factors for the development of lung metastases due to giant cell bone tumors. For that purpose, two groups of patients were compared, based on the literature search, those with and without lung metastases; the latter group was considered the control group. Primary and recurrent tumors were also compared between the two groups, as the recurrent tumor presumably constitutes a risk factor for lung metastasis. Likewise, the recurrence rate was compared between the two groups.

2 Methods

2.1 Patients and Data Collection

The study population consisted of the patients diagnosed with giant cell bone tumors, who were identified in the medical literature during a search conducted in the Embase, Cochrane Central Register of Controlled Trials (CENTRAL), and MEDLINE/PubMed until April 30, 2018. The patients were stratified into those with lung metastases (study group) and without lung metastases (control group). The search for suitable studies was carried out by entering “giant cell tumors of bone” and “lungs” into the search console of the databases. Subsequently, the restrictions “humans” and “abstract available” were applied. The analysis was carried out in line with the recommendations of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Liberati et al. 2009).

Inclusion criteria consisted of the information on gender and age; time period for lung metastases; time interval for recurrences; observation period; classification as a primary or recurrent tumor; possible radiological classification according to Campanacci grade I, II, or III

(Campanacci 1976); localization of the tumor; disease process; and surgical or radiological treatment of primary and recurrent tumors or lung metastases due to giant cell bone tumors. The exclusion criterion was defined as the unmet inclusion criteria outlined above. This meta-analysis included all prospective, retrospective, and evaluation studies, as well as case series and case reports of pulmonary metastases due to giant cell bone tumors in humans.

The inclusion criteria outlined above stem from the clinical knowledge about giant cell bone tumors in humans. Such tumors are more likely to appear in middle-aged women, notably in the third decade of life, and the usual location is near the ends of long bones, notably in the knee joint region, followed by the proximal humerus and the distal radius (Fletcher et al. 2018). These regions were considered together in this review as the lower and upper limbs. Other rare localizations, such as the spine, sacrum, and pelvis also were considered.

The survival probability in patients with giant cell bone tumors, with and without lung metastases, was determined in this review, according to the Kaplan-Meier method, after collection of the number of deaths.

2.2 Assessment of Potential Bias for Study Quality

The purpose of this review was to collect studies that met the inclusion criteria using the Cochrane Collaboration tool to assess a potential risk of bias and thus to reduce bias (Savović et al. 2014). There were 23 (36.5%) retrospective studies, 1 (1.6%) evaluation study, 10 (15.9%) case series, and 29 (46.0%) case reports examined for the review. The risk of bias was assessed in the studies. High risk of bias was regarded for blinding patients and medical personnel and blinding the outcome assessment. Low risk of bias was regarded for valued random sequence generation, incomplete outcome data, and selective reporting. Unclear risk of bias considered the allocation concealment and missing data for the duration of treatment and follow-up Fig. 1.

2.3 Statistical Elaboration

Data were presented as means ±SD and proportions of patients (%). 95% confidence intervals (95%CI) were provided for the proportions of patients in the study and control groups. The mean and median values were calculated to compare age differences, time interval for lung metastases, number of recurrences, time interval for recurrences, and follow-up time.

Odds ratios (OR) with 95%CI were used to determine the relationships between the frequency of lung metastases in the total number of bone tumor patients, gender differences, primary or recurrent tumor classification, death, local primary tumor irradiation, spondylectomy, hemipelvectomy, unknown treatment, embolization, treatment of recurrent tumors by joint or prosthesis replacement or arthrodesis, amputation, excision, lung treatment, local irradiation of recurrences, and the lack of surgery for recurrent tumors.

The Mann-Whitney U test was used to determine the significance of two unpaired distributions of age difference, tumor localization, difference in the number of primary tumors in both patient groups, Campanacci grading, time interval to recurrence, follow-up time, number of recurrences, curettage, resection, amputation, arthrodesis or joint or prosthesis replacement, and the treatment of tumor recurrence by curettage, resection, or by unknown therapy. A one-sample *t*-test was used to calculate the mean time of tumor metastasizing to the lungs, to surgical treatment by excision, assuming a hypothetical value of 1, and to manifestations of osteosarcoma, assuming a hypothetical value of 0.

The results of this meta-analysis were considered significant when a suitable significance test for a given type of data provided a p-value <0.05.

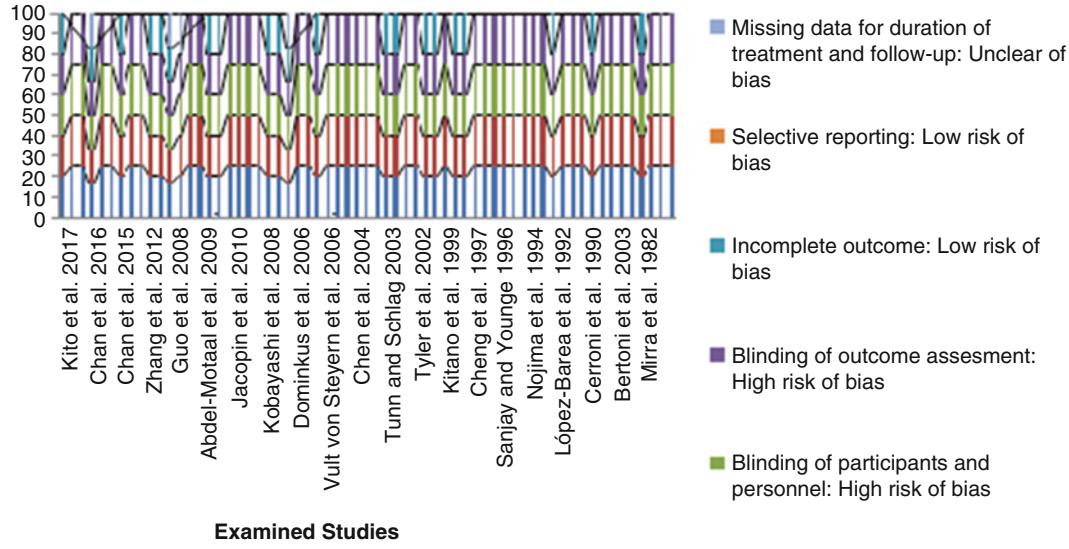

Fig. 1 Valuation of high, low, and unclear biases of risk for the study quality evaluation

3 Aspects of Giant Cell Bone Tumors Addressed in the Literature Searched

3.1 Radiological Classification According to the Campanacci Grading System

Campanacci grading is based on the spread of a tumor over the disease course in all directions within the bone, destruction of the adjacent cortex, and subsequent infiltration of bordering soft tissues. The Campanacci grading system is exclusively based on the evaluation of X-ray images. In grade I, tumor borders are delimited from the environment by a thin rim of mature bone. The cortex is unaffected or slightly diluted, and it is not deformed or broken. In grade II, tumor borders are quite clearly defined without a clear edge defined by mature bone. However, there is a border between the tumor and the surrounding soft tissue. In grade III, the tumor boundaries are blurred, and it extends into the soft tissue (Campanacci 1976).

Typical X-ray of giant cell tumor in a long bone shows eccentric osteolysis, without matrix ossification, and dilution of the bone cortex (Fletcher et al. 2018). In the studies inhere reviewed, X-ray images are used for the diagnosis, followed by computerized tomography (CT) and magnetic resonance imaging (MRI). These examinations also are suitable for tumor staging and surgery planning.

3.2 Time Course of Giant Cell Bone Tumors

The term "primary tumor" was used to describe the first occurrence of a giant cell bone tumor before metastasis. Primary tumors were evaluated in both study and control groups as a potential risk factor for lung metastases. "Tumor recurrence" was referred to as reoccurrence of giant cell bone tumors after complete destruction by surgical removal or radiotherapy, and it also was evaluated as a risk factor for the development of lung metastases. The mean time to tumor recurrence in months was compared between the two groups. "Follow-up" referred to revisiting patients after the last physical examination; it was evaluated in months. Giant cell bone tumors can reoccur after many years, which increases the risk of lung metastases. Therefore, the need for a longer follow-up time was compared between the two groups.

3.3 Pathohistology

The diagnosis was made after taking a biopsy of the primary tumor and performing histological examination of hematoxylin-eosin-stained specimens. Giant cell bone tumor was histologically characterized under light microscopy by mononuclear cells and numerous, diffusely distributed giant cells (Fletcher et al. 2018). In rare cases, giant cell bone tumors could degenerate into highly malignant sarcoma. Such transformation was investigated in this analysis. However, pathohistology served to confirm the diagnosis, suspected on the basis of CT imaging, and it was not the subject of analysis.

3.4 Surgical Treatment

Classical therapy for giant cell bone tumors involves intralesional aggressive curettage, generous opening of the bone cave using a mechanical high-speed milling drill, and physicochemical adjuvants such as bone cement, alcohol, phenol, cryosurgery, or cauterization (Khalil el et al. 2004). These adjuvants were used to reduce a high rate of recurrence of tumors and possible lung metastases. This analysis examined whether surgical technique could reduce the number of lung metastases. For the sake of clarity, individual adjuvants remained undescribed.

En bloc tumor resection is essential for Campanacci grade III aggressive giant cell bone tumors (Pazionis et al. 2013). Radical resection and removal of the actual giant cell bone tumor affect neighboring tissues and lymph nodes. A high probability of recurrence necessitates such radical resection surgery, while a low probability of recurrence may require the curettage only. Due to giant cell bone tumors' ability to invoke hematogenous metastases, difficult cases are treated by extensive amputation (Gupta et al. 2007). This analysis examined whether amputation has been used as a treatment mode in previous studies.

Surgical removal of whole inactive benign giant cell bone tumors is performed in some cases of Campanacci grade I tumors. In advanced cases, accompanied by massive tumor expansion out of the bone and joint involvement, arthrodesis or joint or prosthesis replacement is used as treatment (López-Pousa et al. 2015). In milder cases, marginal excision suffices to remove bone tumor with a margin of surrounding tissue (Guo et al. 2008). Hemipelvectomy is used to remove the whole lower extremities, including one half of the pelvis extending to the sacrum, in cases of severe tumor expansion on one side of the hip and pelvis (Sanjay et al. 1993). Spondylectomy is indicated when the vertebral body is destroyed by a bone tumor, compromising stability and leading to neurological deficits. This procedure involves surgical removal of one or more vertebral bodies with subsequent replacement and stabilization of the spinal column section (Balke et al. 2012; Matsumoto et al. 2007).

Recurrences are sometimes difficult to treat as giant cell bone tumors often reach the articular surface of a bone. Depending on the condition, the intralesional procedure could be repeated. Macroscopically complete removal of a recurring soft tumor mass by curettage is mandatory. The removal should be followed by treatment of the tumor cavity with necrotizing substances and sealing the cavity with bone cement (Xing et al. 2013). For the severe recurrence condition, en bloc resection of a tumor, endoprosthesis, or allografting is recommended (Bergovec et al. 2014). Such procedures are most advantageous for avoiding further growth or infiltration of neighboring tissue (Harris and Lehmann 1983). Incomplete excision of a tumor contributes to increased recurrence rate. If recurrence persists after resection and radiotherapy, amputation of the affected limb is considered (Basu et al. 2012). In the mild condition, recurrence is treated by marginal excision (Xu et al. 2017).

3.5 Radiotherapy

Generally, giant cell bone tumors are irradiated with great success as they are radiosensitive and regress with relatively low irradiation doses. Radiotherapy also is advantageous as it offers substantial possible protection against amputation

and recurrence as after curettage. Indications for radiotherapy include incomplete excision, increased mitotic rate, and pronounced bone involvement.

Irradiation therapy is also considered for giant cell bone tumor recurrences but only as an adjuvant measure when the patient's condition can be hardly controlled by surgery which is the first-line treatment choice for recurrences (Caudell et al. 2003). The possibility of impending limb amputation is another potential indication for radiotherapy. Radiotherapy serves then to reduce tumor mass to keep the area to be amputated as small as possible.

3.6 Embolization

Elective embolization is sometimes useful for controlling difficult giant cell bone tumors. It is performed by radiologically assisted implementation of a liquid plastic substance via a catheter into a patient's artery (Yu et al. 2013).

3.7 Chemotherapy

Adjuvant chemotherapy is proposed for giant cell bone tumors with the intermediate-to-high-grade malignancy after rehabilitative measure. At present, there are no generally accepted and effective chemotherapeutic agents available for treatment of such tumors (Puri and Agarwal 2007). Variable chemotherapeutic regimens have been proposed in the literature, the evaluation of which was beyond the scope of this review.

3.8 Lung Metastases

Giant cell bone tumors could form metastases restricted to the lungs, which should be removed surgically. Solitary lung lesions are usually operable, so that metastasectomy is the method of choice. Surgery enables the histological verification of a diagnosis. Incomplete lung metastasectomy could be performed if metastases are in unreachable locations (Takeuchi et al. 2016; Cheng and Johnston 1997). Pulmonary wedge resection is performed to remove tumorous lung tissue that does not align with the lung anatomical boundaries. Lobectomy is required when there is a widespread metastasis (Muheremu and Niu 2014). Symptomatic, palliative treatment is offered only when no other therapy of lung metastases is possible (Júnior et al. 2016). Lung metastases of giant cell bone tumors sometimes do not show any progressive dynamics of growth and remain of the same size for prolonged periods of time. Some may even spontaneously regress (Kay et al. 1994). In some of the studies, there is no conclusive information on whether surgery was effected or treatment of lung metastases remains unknown. In this review, such cases are referred to as "no lung metastasis surgery" and "unknown", respectively.

In case of problematic or poor resectability of lung metastases, irradiation is an alternative treatment option. However, radiation therapy suffers from the lack of a generally accepted dose or fractionation concept (Roeder et al. 2010).

4 Results

The terms "giant cell tumor of bone" and "lungs" yielded 256 studies in the databases searched. Sixty-three of these studies met the inclusion criteria. Fifty-eight out of the 63 studies were allocated to the study group (giant cell bone tumors with lung metastases), and only were 5 studies allocated to the control group (giant cell bone tumors without lung metastases) (Table 1). In total, 4295 patients were studied, with the study group comprising 247 (5.8%; 95%CI 5.1–6.5%) patients with giant cell bone tumors and lung metastases and the control group comprising 299 (7%; 95%CI 6.2–7.8%) patients without lung metastases. Accordingly, the occurrence of lung metastases increased significantly over the disease course in patients with giant cell bone tumors. Both groups were predominantly male but without appreciable gender or age differences (Table 2).

Table 1 Studies included in this review

Citation	Country	Patients (*n*)	Patients with lung metastases (*n*)	Male (*n*)	Female (*n*)	Mean age (years)
Abdel-Motaal et al. (2009)	Kuwait	1	1	0	1	46.0
Bahri et al. (2003)	Tunisia	1	1	0	1	23.0
Bertoni et al. (2003)	Italy	327	6	5	1	25.3
Bertoni et al. (1988)	USA	97	7	4	3	24.9
Boghani et al. (1994)	India	1	1	0	1	30.0
Cai et al. (2007)	USA	4	4	2	2	27.3
Cerroni et al. (1990)	Austria	1	1	1	0	47.0
Chan et al. (2015)	USA	167	11	5	6	25.3
Chen et al. (2016)	Taiwan	168	7	4	3	39.1
Chen et al. (2004)	Taiwan	1	1	0	1	30.0
Cheng and Johnston (1997)	USA	104	5	2	3	28.6
Dominkus et al. (2006)	Austria	649	14	8	6	27.1
Donthineni et al. (2009)	USA	51	7	4	3	29.9
Erdin and Wegmann (1996)	Switzerland	1	1	1	0	58.0
Faisham et al. (2006)	Malaysia	20	6	5	1	33.7
Feigenberg et al. (2002)	USA	3	3	2	1	27.3
Gresen et al. (1973)	USA	195	2	1	1	57.0
Guo et al. (2012)	China	27	1	0	1	25.0
Guo et al. (2008)	China	16	0	10	6	41.3
Gupta et al. (2008)	India	470	24	15	9	29.1
Hashimoto et al. (2006)	Japan	1	1	0	1	45.0
Hsieh et al. (2012)	Taiwan	1	1	0	1	25.0
Jacopin et al. (2010)	France	1	1	0	1	7.0
Kaiser et al. (1993)	Germany	1	1	0	1	37.0
Kay et al. (1994)	USA	66	6	4	2	28.2
Kitano et al. (1999)	Japan	1	1	0	1	34.0
Kito et al. (2017)	Japan	141	12	9	3	27.0
Kobayashi et al. (2008)	Japan	1	1	1	0	30.0
Kong et al. (2013)	China	79	0	42	37	33.1
Lachat et al. (2004)	Switzerland	1	1	1	0	28.0
López-Barea et al. (1992)	Spain	1	1	1	0	37.0
Maloney et al. (1989)	USA	3	3	1	2	28.0
Mella et al. (1982)	Norway	1	1	0	1	13.0
Miller et al. (2010)	USA	1	1	0	1	29.0
Mirra et al. (1982)	USA	1	1	0	1	45.0
Moon et al. (2012)	South Korea	1	1	1	0	54.0
Muheremu et al. (2015)	China	2	2	1	1	37.5
Nakano et al. (2009)	Japan	1	0	1	0	26.0
Ng et al. (2002)	Malaysia	31	4	2	2	30.2
Nojima et al. (1994)	Japan	1	1	0	1	11.0
Obata et al. (1991)	Japan	1	1	1	0	23.0
Osaka et al. (2004)	Japan	5	5	1	4	21.8
Osaka et al. (1997)	Japan	78	6	3	3	22.3
Powers et al. (1991)	USA	1	1	1	0	45.0
Present et al. (1986)	USA	1	1	1	0	21.0
Qi et al. (2016)	China	12	1	0	1	19.0
Qureshi et al. (2005)	India	1	1	1	0	18.0

(continued)

Table 1 (continued)

Citation	Country	Patients (*n*)	Patients with lung metastases (*n*)	Male (*n*)	Female (*n*)	Mean age (years)
Rock et al. (1984)	USA	31	8	4	4	34.3
Sanjay and Kadhi (1998)	Saudi Arabia	69	3	1	2	22.7
Sanjay and Younge (1996)	Saudi Arabia	1	1	0	1	17.0
Siebenrock et al. (1998)	Switzerland	31	23	11	12	27.0
Tubbs et al. (1992)	USA	475	13	6	7	30.0
Tunn and Schlag (2003)	Germany	87	10	4	6	30.2
Turcotte et al. (2002)	Canada	186	0	90	96	36.0
Tyler et al. (2002)	USA	1	1	1	0	25.0
Viswanathan and Jambhekar (2010)	India	470	23	13	10	26.0
Vult von Steyern et al. (2006)	Sweden	137	1	1	0	21.0
Wan et al. (2012)	China	27	1	0	1	38.0
Yanagisawa et al. (2011)	Japan	11	1	0	1	31.0
Yang et al. (2006)	Taiwan	11	1	0	1	29.0
Yang et al. (2016)	China	17	0	12	5	23.2
Yeo et al. (2015)	Korea	1	1	0	1	22.0
Zhang et al. (2012)	USA	1	1	0	1	43.0

Table 2 Demographic and clinical data of patients with giant cell bone tumors with (study group) and without (control group) lung metastases

Giant cell bone tumor	Study group ($n = 247$)	Control group ($n = 299$)	*p*-value; OR (95%CI)
Male; *n* (%)	129 (52.2)	155 (51.8)	
Female; *n* (%)	118 (47.8)	144 (48.2)	0.928; 1.02 (0.72–1.42)
Patients' age			
Mean age ± SD; years	29.6 ± 9.6	31.9 ± 6.6	0.559
Median (range); years	28.6 (7–58)	33.1 (23.2–41.3)	
Time to lung metastases			
Mean ± SD; months	38.2 ± 52.8	–	**<0.0001;** (24.01–52.30)
Median (range); months	23.8 (0–360)	–	
Time to recurrence			
Mean ± SD; months	19.8 ± 17.2	23.5 ± 10.6	0.351
Median (range); months	12.8 (2–84)	23 (12–36)	
Follow-up time			
Mean ± SD; months	80.4 ± 61.1	123.9 ± 130.2	0.792
Median (range); months	71.7 (1.3–360)	58.8 (51.6–384)	
Tumors, n (%)			
Primary	79 (32.0)	165 (55.2)	0.406
Recurrent	168 (68.0)	134 (44.8)	**<0.0001;** 2.62 (1.84–3.72)
Campanacci grade; *n* (%)			
I	6 (2.4)	8 (2.7)	1.0
II	33 (13.4)	146 (48.8)	**0.028**
III	115 (46.6)	142 (47.5)	**0.006**
Unknown	93 (37.7)	3 (1.0)	**<0.0001**
Localization of tumors; *n* (%)			
Lower limb	139 (56.3)	225 (75.3)	**0.0007**

(continued)

Table 2 (continued)

Giant cell bone tumor	Study group ($n = 247$)	Control group ($n = 299$)	p-value; OR (95%CI)
Upper limb	70 (28.3)	74 (24.7)	0.129
Spine	16 (6.5)	0	0.274
Sacrum	14 (5.7)	0	0.051
Pelvic	8 (3.2)	0	0.356
Disease course; n (%)			
Sarcoma	13 (5.3)	0	0.073
Death	37 (15.0)	0	**0.001;** 106.7 (6.5-174.5)

OR (95%CI), odds ratio with 95% confidence interval; significant p-values are in bold

The time to the occurrence of lung metastases significantly differed among patients with giant cell bone tumors ($p < 0.0001$). Sometimes, lung metastases were found at the time of diagnosis of the primary tumor, but occasionally they occurred during the disease course some years later. The time to tumor recurrence did not differ between the patients with and without lung metastases. Nor was the follow-up period different between the two groups of patients (Table 2).

There was no difference in the number of primary giant cell bone tumors diagnosed in the two groups of patients. However, patients with lung metastases had a significantly greater proportion of recurrent tumors ($p < 0.0001$). In both groups of patients, there was a significantly greater rate of Campanacci grade II ($p = 0.028$) and III ($p = 0.006$) tumors according to the radiological classification of tumors. Giant cell bone tumors were significantly more often localized in the lower extremities ($p = 0.0007$). Osteosarcoma was occasionally detected histologically in patients with bone tumors and lung metastases, but not in those without lung metastases. Death rate was significantly greater in patients with bone tumors and lung metastases ($p = 0.001$) (Table 2).

In patients with primary giant cell bone tumors and lung metastases, curettage was performed significantly less often than in those without lung metastases ($p = 0.0005$). In contradistinction, local radiation was performed more often in patients with lung metastases ($p = 0.008$). Surgery apparently tended to be shunned or not undertaken in these patients. Interestingly, radiographic endovascular embolization did not play an appreciable role in treatment of patients with primary tumors and lung metastases (Table 3).

En bloc resection was the most frequent surgical procedure in patients in the control group who had recurrent giant cell bone tumors without lung metastases ($p = 0.004$), which was followed by curettage. In patients with lung metastases, joint or prosthesis replacement, arthrodesis, limb amputation, and local radiotherapy predominated (Table 3).

Treatment of lung metastases in patients with giant cell bone tumors depended on the extent of metastases. Surgical treatment included complete resection, wedge resection, incomplete resection, and, less often, lobectomy. Symptomatic treatment also was considered an important component of a comprehensive treatment plan, particularly in case of progressive lung metastases of giant cell bone tumors (Table 4).

For unknown reasons, in a few studies reviewed, it was decided not to surgically treat lung metastases by giant cell bone tumors. In hopeless cases, lung irradiation was promising and used in some cases of lung metastases tumors. Due to tumor progression, chemotherapy was used in nearly one-third of the cases of lung metastases; all these interventions were with statistical significance (Table 4).

There were 37 (15%) deaths in the study group of giant cell bone tumors with lung metastases. The mortality risk was increased due to giant cell bone tumors with an OR of 106.7 (95%CI 6.5–174.5%) ($p = 0.001$) (Table 2). Survival probability in the study group was 85.0% (95% CI 80.2–89.8%) according to the Kaplan-Meier method.

Table 3 Treatment of primary and recurrent giant cell bone tumors in patients with (study group) and without (control group) lung metastases

	Study group	Control group	
	$n = 247$	$n = 299$	p-value
Primary giant cell bone tumors			
Surgical treatment; *n* (%)			
Curettage	101 (40.9)	253 (84.6)	**0.0005**
En bloc resection	48 (19.4)	41 (13.7)	0.061
Arthrodesis, joint or prosthesis replacement	19 (7.7)	1 (0.3)	0.582
Amputation	31 (12.6)	4 (1.3)	0.206
Hemipelvectomy	5 (2.0)	0	0.078
Marginal excision	4 (1.6)	0	0.500
Spondylectomy	2 (0.8)	0	0.244
No surgery; *n* (%)	5 (2.0)	0	0.078
Unknown; *n* (%)	5 (2.0)	0	0.078
Local irradiation; *n* (%)	17 (6.9)	0	**0.008**
Embolization; *n* (%)	2 (0.8)	0	0.244
Recurrent giant cell bone tumors			
Surgical treatment of recurrent tumors; *n* (%)			
Curettage	31 (18.5)	37 (27.6)	0.089
En bloc resection	17 (10.1)	69 (51.5)	**0.004**
Arthrodesis, joint or prosthesis replacement	26 (15.5)	0	**0.006**
Amputation	17 (10.1)	0	**0.017**
Marginal excision	3 (1.8)	0	0.252
Spondylectomy	1 (0.6)	0	0.591
No surgery; *n* (%)	1 (0.6)	0	0.591
Unknown; *n* (%)	58 (34.5)	28 (20.9)	0.180
Local irradiation of recurrent tumors; *n* (%)	15 (8.9)	0	**0.022**

Significant p-values are in bold

Table 4 Treatment of lung metastases in patients with giant cell bone tumors (study group)

Treatment	Study group ($n = 247$)	p-value
Surgical; *n* (%)		
Complete metastasectomy	88 (35.6)	**0.0001**
Wedge resection	36 (14.6)	**0.001**
Incomplete metastasectomy	15 (6.1)	**0.010**
Lobectomy	7 (2.8)	**0.045**
Symptomatic; *n* (%)	24 (9.7)	**0.004**
Observation; *n* (%)	14 (5.7)	**0.012**
Lack of surgery; *n* (%)	29 (11.7)	**0.002**
Unknown; *n* (%)	5 (2.0)	0.078
Refusal of treatment; *n* (%)	6 (2.4)	0.059
Lung radiation; *n* (%)	23 (9.3)	**0.004**
Chemotherapy; *n* (%)	80 (32.4)	**0.0001**

Significant p-values are in bold

5 Discussion

The present review of the literature demonstrates that giant cell bone tumors increase the risk of lung metastases. The probability of being inflicted with lung metastases amounts to 39.5%. The percentage of patients with pulmonary metastases is 5.8%, and the frequency of lung metastases varies from 1% to 6% (Tubbs et al. 1992). Large fluctuations in the time to the development of lung metastasis were noted in the publications. Lung metastases were present in only four (1.6%) patients at diagnosis (Moon et al. 2012; Jacopin et al. 2010; Bahri et al. 2003; Nojima et al. 1994). One patient exhibited lung metastasis 30 years after the first presentation (Erdin and Wegmann 1996). Such extremely divergent time intervals show how giant cell bone tumors can be unpredictable.

The probability of lung metastases increases when there is a local tumor recurrence. The recurrence rate in this meta-analysis was 68.0%, the highest reported amounted to 82.6% (Siebenrock et al. 1998), among patients with lung metastases and 44.8% among those without lung metastases. Yang et al. (2017) have come to the same conclusion, finding a local recurrence rate of 73.9% and a correlation between local recurrence and lung metastasis. Therefore, performing careful therapy and ongoing controls at regular intervals to prevent local recurrence is of great importance.

The Campanacci radiological classification of giant cell bone tumors describes a tumors' tendency to expand beyond the cortical bone and to destroy it (Campanacci 1976). This classification is based on the projected radiographic appearance of the bony lesion. In conventional X-rays, giant cell bone tumors are predominantly observed as expansive, transparent, and osteolytic bone lesions in the epiphysis. The classification stratifies the tumors into three grades, with the predominance of grade II and III lesions (Panzica et al. 2014). These higher Campanacci grades have been reported to be risk factors for progression of lung metastasis (Kito et al. 2017; Yang et al. 2016; Muheremu et al. 2015; Faisham et al. 2006). A majority of giant cell tumors are classified as grade II or III at the first diagnosis. Therefore, the use of the Campanacci classification is essential to foretell the likelihood of lung metastases due to giant cell bone tumors.

Giant cell bone tumors are most commonly located in the lower extremities, such as in the distal femur and proximal tibia. The upper extremities, such as the distal radius, come in second place of localization frequency (Muheremu and Niu 2014). In the present meta-analysis, giant cell bone tumors were mostly identified in the lower extremities. The presumption might be that the location of a primary tumor at the axial skeleton could lead to more lung metastases due to the immediate proximity of the lungs to the skeleton. However, these locations are less frequently invaded by tumors than the limbs are. Therefore, primary tumor localizations do not appear a worthwhile prognostic of the development of lung metastases.

Giant cell bone tumors may transform into malignant sarcoma. The frequency of osteosarcoma amounted to 5.8% in the studies inhere reviewed, which was rather high. In contrast, Bertoni et al. (2003) have reported a 1.8% frequency of osteosarcoma. The incidence of osteosarcoma also has been smaller in some other previous studies (Miller et al. 2010; Donthineni et al. 2009; Hashimoto et al. 2006; Lachat et al. 2004; Mella et al. 1982).

Giant cell bone tumors are usually benign (Gresen et al. 1973). They may, rarely, become malignant, leading to death. As with any other tumor-based disease, giant cell bone tumors may lead to reduced life expectancy when they spread to other parts of the body (Amanatullah et al. 2014; Tunn and Schlag 2003). In this meta-analysis, death was more common in patients with giant cell bone tumors that metastasized to the lungs. Seven (18.9%) of such cases were caused by osteosarcoma. The remaining 30 (81.1%) deaths were during the disease course that was uncomplicated by lung metastases.

Since giant cell bone tumors constantly grow larger and constrict the surrounding tissue, such as tendons and joints, they ought to be removed by surgery. Currently, surgical removal of a primary giant cell bone tumor offers the only chance

of recovery, although there is a high rate of recurrence, which, in turn, is associated with increased risk of metastatizing to the lungs (Rigollino et al. 2017). The method of choice for locally aggressive giant cell bone tumors is curettage and implantation of bone cement (Stan et al. 2016). However, single curettage with spongy filling is bound to a high rate of recurrence, so that a careful treatment of the tumor cavity with necrotizing substances, such as phenol, and blinding the cavity is preferred (Fraquet et al. 2009). Extensive, radical surgical treatments, such as en bloc resection or, in the most severe cases, amputation, were used less frequently to treat primary tumors. Recurrent giant cell bone tumors may be retreated by curettage, using a high-speed bur in combination with adjuvant treatment. Again, curettage was the most common surgery performed to treat recurrent tumors in patients with lung metastases, belonging to the study group in this review, although the choice of this treatment option failed to achieve statistical significance. The next most common treatments were en bloc resection, arthrodesis, joint or prosthesis replacement, and amputation.

Small lung lesions can be removed by complete lung metastasectomy. This was the most commonly performed lung metastasis surgery found in the literature review (see, e.g., Kito et al. 2017; Chen et al. 2016; Muheremu et al. 2015; Chan et al. 2015; Guo et al. 2012). Larger lung metastases had to be removed by wedge resection, which was the second most common surgical technique found (see, e.g., Yeo et al. 2015; Cai et al. 2007; Dominkus et al. 2006; Feigenberg et al. 2002; Erdin and Wegmann 1996). Incomplete resection of lung metastases took place rather rarely in cases of unfavorable localization or multiple metastases (see, e.g., Kito et al. 2017; Dominkus et al. 2006; Siebenrock et al. 1998; Kay et al. 1994; Rock et al. 1984). Lobectomy was unavoidable in only a few cases (see, e.g., Hsieh et al. 2012; Dominkus et al. 2006; Hashimoto et al. 2006; Kitano et al. 1999; Tubbs et al. 1992). Symptomatic treatment was more often performed when lung metastases were inoperable or when surgical treatment was refused (see, e.g., Muheremu et al. 2015; Viswanathan and Jambhekar 2010; Gupta et al. 2008; Faisham et al. 2006; Ng et al. 2002). There was a spontaneous regression of lung metastases noticed in a minority of patients, which was subject of observation rather than surgery (see, e.g., Kito et al. 2017; Chen et al. 2016; Chan et al. 2015; Yanagisawa et al. 2011; Abdel-Motaal et al. 2009). Surgical removal of lung metastases was not equally suitable for all patients. Extensive pulmonary metastases were often associated with alleviation of symptoms and, for various reasons, tumor-specific surgical therapy had to be abandoned.

Radiation therapy to compensate for incomplete curettage or excision for therapeutic or functional motives was commonly recommended for very aggressive tumors, especially in those localized to the spinal column (Sobti et al. 2016). Pulmonary irradiation was used to compensate for incomplete lung metastasectomy or to treat uncontrollably progressing metastases (Bennett Jr et al. 1993). Local irradiation of the primary tumor had to be performed in some cases (see, e.g., Chan et al. 2015; Donthineni et al. 2009; Gupta et al. 2008; Tyler et al. 2002; Siebenrock et al. 1998). Somewhat more frequently, recurrent tumors had to be irradiated (see, e.g., Donthineni et al. 2009; Hashimoto et al. 2006; Osaka et al. 2004; Lachat et al. 2004; Ng et al. 2002). The relationship of secondary sarcomatous transformation to the area of radiation-treated giant cell bone tumors has been discussed in the past (Mondal et al. 2002).

Chemotherapy is administered mainly to treat progression and malignant transformation of lung metastases. It was used in almost one-third of cases with lung metastases due to giant cell bone tumors in the reviewed literature (see, e.g., Kito et al. 2017; Chen et al. 2016; Chan et al. 2015; Moon et al. 2012; Viswanathan and Jambhekar 2010). It is worthwhile to note that different chemotherapy regimens were employed. However, evaluation of the effectiveness of individual regimens was beyond the scope of this review. Currently, there are no generally accepted chemotherapy regimens for treatment of giant cell bone tumors (Sobti et al. 2016).

There are studies indicating that women are more likely than men to suffer from giant cell bone tumors (Tunn and Schlag 2003). Likewise,

women are more likely to form lung metastases (Estrada-Villaseor et al. 2015). In the present review, however, the findings were opposite in that more men than women were found to have lung metastases. In addition, the gender difference was small and without statistical significance.

Giant cell bone tumors occur most commonly in the third and fourth decades of life (Tunn and Schlag 2003). That was confirmed in the present review as the mean age at diagnosis ranged from 29.6 to 31.9 years, for the patients with and without lung metastases, respectively, which made no significant difference ($p > 0.05$). A previous study by Estrada-Villaseor et al. (2015) has examined the patients' age, among other clinical and pathological aspects, in relation to the development of lung metastases due to giant cell bone tumors, compared to other tumors. The mean patients' age reported in that study has been 36 ± 16 years, with no significant difference to metastases, which is grossly in line with the present findings. It appears, therefore, that age at diagnosis of giant cell tumors is not a potential risk factor for the capacity to develop lung metastases.

In conclusion, there is increased likelihood of lung metastases in patients with giant cell bone tumors, even a long time after the initial occurrence of tumor. The probability of lung metastases increases in recurrent tumors and in severe cases of the Campanacci grade II and III tumors. However, randomized controlled studies of the risk factors for lung metastases due to giant cell bone tumors are missing, which might be due to the rarity of this type of bone tumor as well as for ethical reasons. Giant cell bone tumors favor the lower extremities. The tumors are most often described as being benign, but they may occasionally become fatal. Care should be paid to the disease progress and to the follow-up after curettage treatment. Generally, the long-term follow-up of many years is recommended for the giant cell bone tumors.

Conflicts of Interest The author declares no conflicts of interest in relation to this article.

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Acceptance of Illness Associates with Better Quality of Life in Patients with Nonmalignant Pulmonary Diseases

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Abstract

Chronic nonmalignant pulmonary diseases impose a heavy burden on patients, generate health-care costs, and contribute to poor health-related quality of life. It has been found that a wide range of factors negatively affects quality of life, but the role of acceptance of illness needs to be further investigated. The aim of the study was to evaluate the relationship between acceptance of illness and quality of life in patients with chronic nonmalignant pulmonary diseases. The study encompassed 200 patients of the mean age 58 ± 16 years who were mainly diagnosed with asthma (n = 72; 36%), COPD (n = 52; 26%), and obstructive sleep apnea (n = 38; 19%). The patients answered the Acceptance of Illness Scale (AIS) and the St. George's Respiratory Questionnaire (SGRQ). Sociodemographical and clinical data were collected. The level of acceptance of illness significantly associated with each of the SGRQ domains. The greater the acceptance of illness, the lowest was the SGRQ score. The mean total score of SGRQ was 44.6 ± 24.9 and that of AIS was 26.1 ± 8.2. Higher AIS scores significantly associated with lower SGRQ scores, i.e., with better quality of life ($p < 0.001$ for each domain). We conclude that in patients with chronic nonmalignant pulmonary diseases, acceptance of illness plays an important role and is closely related to the general level of quality of life. Interventions aimed at improving acceptance of illness may be considered to improve quality of life.

Keywords

Acceptance of illness · Asthma · Obstructive sleep apnea · Pulmonary diseases · Quality of life

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1 Introduction

Chronic nonmalignant pulmonary diseases impose a heavy burden on patients, their caregivers, and the health-care system in terms of their impact on

the economic condition and quality of life (Lewis et al. 2016). The review performed by Bahadori et al. (2009) showed that asthma is associated with high health-care costs which include hospitalizations and medications as well as work and school loss. Similar to asthma, severe stages of chronic obstructive pulmonary disease (COPD) generate high costs due to hospitalizations and medications, while a mild stage of COPD is associated with productivity loss due to sick leave and early retirement (Jansson et al. 2013). Tarasiuk and Reuveni (2013) reported that obstructive sleep apnea is associated with increased medical costs as well. Further, the sickest tertile of patients consume 65–82% of all medical expenses. Increased medical costs in chronic pulmonary diseases limit patient employability and substantially worsen their financial condition. Economic consequences of the disease along with pulmonary symptoms impair the health state and translate into poor health-related quality of life (Srivastava et al. 2015).

The assessment of health-related quality of life is important for patients with chronic pulmonary diseases as poor quality of life is associated with poor outcomes (Wang and Bourbeau 2005; Domingo-Salvany et al. 2002). Literature reports confirm that there is a wide range of factors that negatively affect quality of life. They include smoking, quality of inhaled air, increased body weight, exacerbations and hospitalizations, advanced stage of the disease, older age, and comorbidities, e.g., cardiovascular diseases, depression, anxiety, or upper respiratory tract infections (Nakao et al. 2018; Sundh et al. 2017; Yoo et al. 2016). Factors that increase quality of life include higher education, knowledge about self-management of exacerbations, health-promoting physical activity, and better symptom control (Uchmanowicz et al. 2016; Gonzalez-Barcala et al. 2012). The relationship between the acceptance of illness and quality of life has not been established, although some studies address this problem among patients suffering from other than chronic pulmonary diseases. Therefore, the aim of this study was to evaluate the level of acceptance of illness and quality of life in patients with nonmalignant pulmonary diseases and to determine the relationship between the acceptance of illness and quality of life in this group of patients.

2 Methods

Two hundred patients were enrolled into the study. All the patients underwent treatment for a chronic pulmonary disease between January 2017 and October 2017 at the Department of Pulmonology and Lung Cancer of Wroclaw Medical University in Wroclaw, Poland. They were asked to fill out the Acceptance of Illness Scale (AIL), in the Polish adaptation by Jurczyński (2009), and the St. George's Respiratory Questionnaire (SGRQ). The sociodemographical and clinical data also were collected. The AIS instrument assesses limitations caused by the illness, lack of independence, self-perceived feeling of being dependent on others, and changes in self-esteem (Felton et al. 2001; Felton and Revenson 1984). Overall, the scale assesses the degree of acceptance of illness, with the higher score pointing to better acceptance. The scale contains eight statements followed by a 5-choice Likert-type rating. The respondent indicates the level of agreement in the following way, 1 = strongly agree, 2 = agree, 3 = undecided, 4 = disagree, and 5 = strongly disagree, with a sum ranging from 8 to 40 points. A score of $\leq$18 denotes the lack of illness acceptance, and $\geq$ 30 denotes a high level of illness acceptance. The Polish version of AIS has a high Cronbach's reliability of $\alpha = 0.82$ (Rogon et al. 2017).

The SGRQ questionnaire is a self-administered, disease-specific instrument, commonly used for the evaluation of quality of life, including overall health, daily life, and perceived well-being of patients with obstructive airway diseases (Jones et al. 1991). There are 50 items divided into three components (symptoms, 8 items; activity, 16 items; and impacts, 26 items), which are scored separately. Scores range from 0 (no impairment) to 100 (maximum impairment) (Jones et al. 1992). The questionnaire validation performed for the British patients with bronchiectasis shows a high intra-class correlation for the short-term

repeatability. The correlation coefficients are 0.93 for the symptoms, 0.98 for the activity, 0.94 for the impacts, and 0.97 for the total score. Cronbach's reliability is $\alpha = 0.90$ for the symptoms, 0.89 for the activity, and 0.92 for the impacts (Wilson et al. 1997). The SGRQ has also been validated among the Polish patients with bronchial asthma, with Cronbach's reliability of $\alpha = 0.75$ for the total score and all subscale scores (Kuzniar et al. 1999).

Quantitative data from questionnaires were expressed as means ±SD and range and medians and interquartile range (IQR). Categorical data were expressed in counts and percentages. Data distribution was checked with the Shapiro-Wilk test. Spearman's rank correlation coefficient was used to assess the correlation between the domains of health status and the illness acceptance. The R free software v3.4.2 was used for all analyses.

Table 1 Characteristics of the patients investigated ($n = 200$)

Variable		*n*	%
Gender	Men	102	51.0
	Women	93	46.5
	Missing data	5	2.5
Place of residence	City	123	61.5
	Rural area	75	37.5
	Missing data	2	1.0
Education	Primary	33	16.5
	Vocational	52	26.0
	Secondary	56	28.0
	University	59	29.5
Work activity	Employed	77	38.5
	Unemployed	19	9.5
	Pensioner	102	51.0
	Missing data	2	1.0
Smoking status	Current smokers	55	27.5
	Never-smokers	69	34.5
	Past smokers	76	38.0

3 Results

The analysis was based on questionnaires collected from all 200 patients of the mean age of 58 ± 16. The patients' diagnoses were chronic obstructive or restrictive airway diseases, including asthma (n = 72; 36%), COPD ($n = 52$; 26%), and obstructive sleep apnea ($n = 38$; 19%). Pneumonia or bronchitis of various etiologies was diagnosed in 17 (8.5%) patients. Additionally, there were patients with obstructive pulmonary symptoms in the course of treatment of a cancer disease ($n = 10$; 5%), interstitial lung disease ($n = 6$; 3%), and bronchiectasis ($n = 5$; 2.5%). The majority of the patients lived in the urban area and were employed. Only did about one-third of the patients belong to never-smokers. The characteristics of the study population are summarized in Table 1.

The mean score of AIS was 26.1 ± 8.0 and that of SGRQ was 44.6 ± 24.9 (Table 2). The level of illness acceptance associated significantly with each SGRQ domain. The greater the acceptance, the lower was the SGRQ score, i.e., the better quality of life. The strongest association occurred between the level of illness acceptance, on the one side, and the overall SGRQ score and the impact on life quality score, on the other side. Coefficients of the relationships are shown in Table 3.

4 Discussion

In the present study performed in patients with chronic pulmonary diseases, the overall quality of life measured with a disease-specific questionnaire is low. Patients scored an average of 44 out of the 100 points possible. The level of illness acceptance was moderate, an average of 26 out of the 40 points. We also found a significant association between the level of illness acceptance and quality of life, indicating that the acceptance of illness plays a key role in maintaining a reasonable quality of life, and as such, the level of illness acceptance may contribute to medical outcome.

It has been shown that patients with chronic diseases have reduced or moderate acceptance of illness. A study of Kupcewicz and Abramowicz (2015), which encompassed patients with COPD, has identified the following determinants of illness acceptance: the presence of comorbidities, time from the diagnosis, gender, age, hospitalization rate, and social and work status. Another

Table 2 Results of the St. George's respiratory questionnaire and the acceptance of illness scale

scale	n	mean ± sd	range	median	IQR
AIS	200	26.1 ± 8.2	8.0–40.0	27.0	19.8–32.0
SGRQ					
Total score	198	44.6 ± 24.9	0–96.5	41.8	26.3–61.8
Symptoms	198	53.0 ± 25.3	0–97.6	54.9	34.7–73.3
Activity	200	51.8 ± 29.4	0–100.0	53.5	29.6–72.8
Impacts	200	37.7 ± 25.9	0–95.8	34.9	17.5–57.2

SGRQ St. George's Respiratory Questionnaire, *AIS* Acceptance of Illness Scale, *IQR* interquartile range

Table 3 Associations between the scores of Acceptance of Illness Questionnaire and St. George's Respiratory Questionnaire domains

	SGRQ			
AIS	Total score	Symptoms	Activity	Impact
	−0.708∗	−0.549∗	−0.646∗	−0.712∗

SGRQ St. George's Respiratory Questionnaire, *AIS* Acceptance of Illness Scale; $*p < 0.001$

study comparing patients with diabetes reveals that illness acceptance worsens over time, possibly with disease progression (Rogon et al. 2017). In that study, no gender-related difference in illness acceptance has been noticed in patients up to 65 years of age. The lack of illness acceptance is reported by 54.1% and 54.5% of men and women, respectively, but the number of patients who cannot accept the disease increases over time considerably. The peak increases, however, differ. They amount to 14.7% for men and to 33.7% in women, the latter being significant ($p < 0.05$).

The association between illness acceptance and quality of life has been evaluated in a few studies among patients with other chronic diseases which pose a significant burden on patients. Denys et al. (2015) have examined a group of 105 patients with osteoarthritis of the hip and knee. They find that a higher level of quality of life, as measured with SF-36 questionnaire, associates with a greater illness acceptance. Additionally, quality of life is higher in patients who have a better pain control. Riedl et al. (2015) have investigated quality of life in patients with a different degree of acceptance of chronic tinnitus. That study shows that patients with moderate-to-high acceptance of tinnitus have significantly higher quality of life and lower psychological distress than those with low-to-mild acceptance of tinnitus. Another study in 111 patients of the stoma clinic performed by Zhang et al. (2013) has revealed similar associations. In patients who had undergone colostomy at least 1 month before answering questionnaires, general health and quality of life were significantly associated with the acceptance of disability and all of its dimensions. In yet another study conducted by Obieglo et al. (2016) in a group of 100 patients with chronic heart failure, illness acceptance is an independent predictor of quality of life as measured with the Nottingham Health Profile. The authors conclude that greater acceptance of the diagnosis of chronic heart failure is associated with greater quality of life.

Only have a few studies addressed the population of patients with pulmonary diseases which seems to be insufficient to confirm the association between quality of life and acceptance of illness in general. Jankowska-Polanska et al. (2016) have investigated the effect of illness acceptance on quality of life in 105 patients with COPD. The authors show that in patients who scored ≥29 points on the Acceptance of Illness Scale, they have a significantly higher general level of quality of life and all of its domains. The study carried out by Polanski et al. (2018) in 257 patients with lung cancer shows that the diagnosis of small cell lung cancer is associated with a lower level of quality of life and illness acceptance in comparison to non-small cell lung cancer. It is worth noting that health status of patients with small-cell lung cancer is significantly worse than that of patients with non-small cell lung cancer. The results of the present study are in line with those previous reports that included patients with lung

diseases as well as with other chronic diseases. We confirmed the presence of an association between quality of life and illness acceptance.

In previous studies, it has been shown that the determinants of quality of life and illness acceptance also include the severity of symptoms, disease progression, psychological distress, and the type of disease (Polanski et al. 2018; Denys et al. 2015; Riedl et al. 2015). Therefore, an inherent aptitude concerning the disease control, which involves the education of patients on how to cope with exacerbations or how to properly use medications and devices such as inhalers, can help improve the patients' quality of live (Uchmanowicz et al. 2016; Virchow et al. 2015).

In conclusion, in patients with chronic pulmonary diseases, acceptance of illness plays an important role and is closely related to the general level of quality of life. Interventions aimed at improving acceptance of illness may be considered as also improving quality of life. The ability to exercise control over disease symptoms helps improve both acceptance of illness and quality of life.

Conflicts of Interests The authors declared no conflicts of interest in relation to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of Wroclaw Medical University in Poland (permit 32/2017).

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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Heart Rate Variability in the Diagnostics and CPAP Treatment of Obstructive Sleep Apnea

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Abstract

Obstructive sleep apnea (OSA) is the most common manifestation of sleep-related breathing disorders that are often accompanied by dysfunction of the autonomic nervous system. The main objective of the study was to assess the usefulness of heart rate variability (HRV) analysis in the diagnosis of patients with severe OSA and in the assessment of the effects of 3-month treatment with continuous positive airway pressure (CPAP). There were 54 patients enrolled in the study. The OSA group consisted of 39 patients suffering from severe OSA (apnea/hypopnea index >30/h), and the control group included 15 non-OSA patients with matched demographic characteristics and comorbidities. All patients underwent 24-h Holter electrocardiographic monitoring. HRV was analyzed using the time- and frequency-domains. We found that OSA patients had decreases in time-domains and increases in frequency-domains of HRV, compared to non-OSA controls, which strongly suggested a clinically disadvantageous shift in the balance of parasympathetic/sympathetic activity toward the latter. Further, CPAP treatment, partly, albeit significantly, reversed the OSA-induced changes in HRV. We conclude that HRV analysis may be of help in the diagnosis of OSA and in the monitoring of the effectiveness of treatment.

Keywords

Autonomic nervous system · Continuous positive airway pressure · Heart rate variability · Obstructive sleep apnea · Polysomnography

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1 Introduction

Obstructive sleep apnea (OSA) is a chronic disease and the most frequent type of sleep-related breathing disorders. It is characterized by obstruction of the upper airway despite ongoing breathing efforts. This most frequently leads to a fall in hemoglobin oxygen saturation and awakenings (AASM 2005). It is estimated that the syndrome is present in approximately 5% of the general human population (Jennum and Riha 2009; Pływaczewski et al. 2008). If untreated, OSA can lead to a number of severe medical conditions with a prevalence of cardiovascular episodes (Somers et al. 2008;

Marin et al. 2005). Polysomnography is the gold standard in the diagnosis of sleep breathing disorders, while the use of continuous positive airway pressure (CPAP) is the standard OSA treatment (Pataka and Riha 2013; Buchner et al. 2007).

The autonomic nervous system (ANS) plays a key role in the regulation of the physiological processes taking place during sleep. ANS activity is reflected in changes in arterial blood pressure, respiratory tract function, muscle tension, and heart rate variability (HRV). The HRV is defined as the variability of RR intervals measured electrocardiographically (ECG). It is affected by a number of factors, both physiological, e.g., breathing, physical, or psychological activity, and pathological such as diseases involving ANS dysfunction or medications. HRV analysis in time- and frequency-domains is a noninvasive tool for the evaluation of ANS activity. The time-domain provides the information on the RR interval length. The physiological heart beats generated in the sinus node are defined as normal (N) and the RR interval as normal-to-normal beats (NN). The commonly used time-domain parameters are the following: standard deviation of NN intervals (SDNN), standard deviation of the average NN intervals (SDANN), and the square root of the mean squared successive differences of NN intervals (RMSSD). A decrease in SDNN points to a possible loss of normal circadian variation in the length of NN intervals. This is observed in the condition of enhanced sympathetic activity. The RMSSD reflects the variability in the length of successive NN intervals, which is fundamentally affected by the vagal nerve parasympathetic activity (Reynolds et al. 2007; Bernardi et al. 2000; Malik and Camm 1993).

The frequency-domain of HRV assesses changes in the length of NN intervals in a given unit of time. The spectrum of NN interval frequency is obtained from the Fourier transformation method. The European Society of Cardiology recommends the spectral power analysis of NN intervals be made in the following frequency ranges: ultralow, lower than 0.0033 Hz (ULF); very low, 0.003–0.040 Hz (VLF); low, 0.04–0.15 Hz (LF); high, 0.15–0.40 Hz (HF); and total spectral power (TP). The spectral power in given frequency band provides the information on the cardiovascular modulation by specific components of ANS. Vagal nerve activity is represented by the HF band. The degree of both sympathetic and parasympathetic activity involved in the baroreceptor reflex mechanism is represented by LF band. Parasympathetic activity is represented by the ratio of LF to HF spectrum. The interpretation of ULF and VLF bands is somehow less clear. The VLF spectrum presumably reflects the parasympathetic activity, as it is inhibited by atropine and the ULF spectrum may reflect the circadian variations in HRV (Sun et al. 2011; ESC 1996).

There is ample evidence showing that the pathophysiological events occurring during sleep in OSA patients impair the ANS function (Xie et al. 2017; Tobaldini et al. 2013; Flevari et al. 2015; Karasulu et al. 2012; Stein and Pu 2012; Gula et al. 2003). Blood pressure variations in the chest cavity, changes in heart load, and apneic episodes and multiple awakenings at night tend to shift the vagosympathetic balance toward the latter component. A growing health issue of OSA, with the syndrome's socioeconomic implications, calls for a further exploration of the pathophysiological mechanisms involved. Polysomnography remains a highly specialized diagnostic procedure, with a rather limited general accessibility. Therefore, other effective OSA screening and diagnostic tools are searched for. The HRV analysis, based on ECG Holter monitoring, has been proposed as such a tool (Gong et al. 2016; Harrington et al. 2013; Hayano et al. 2013; Roche et al. 1999b). Therefore, the present study seeks to define to what extent the HRV analysis could be useful in the diagnosis of OSA and in the assessment of CPAP treatment efficacy.

2 Methods

2.1 Patients, Clinical Tests, and Study Protocol

There were two groups of patients in this study: 39 (29 men and 10 women) middle-aged OSA patients, who manifested severe symptoms of the

disease (AHI ≥30 episodes/h), and 15 control patients (10 men and 5 women) free of any sleep-related breathing disorders. Both groups of patients were matched regarding the age, gender, and a history of hypertension. The BMI was considerably higher in the OSA patients (Table 1). The OSA patients qualified for the study were chosen from the initial cohort of 140 patients who were at onset considered as the potential candidates for the study. The qualification and exclusion criteria for the study are listed in Table 2. All patients underwent the baseline evaluation consisting of medical examination and measurement of blood pressure, blood oxygen saturation, body mass index (BMI), ECG, sleepiness on the Epworth sleepiness scale (ESS), basic blood tests (morphology; glucose level; lipids; sodium, potassium, and magnesium levels; creatinine; N-terminal pro b-type natriuretic peptide (NT-proBNP); alanine transaminase; bilirubin; total protein; C-reactive protein; and thyrotropin-stimulating hormone), spirometry, and a chest X-ray.

Polysomnography and 24-h ECG Holter monitoring were conducted in all the subjects. In the group of OSA patients, CPAP treatment was initiated. Therapeutic CPAP pressure values were defined during a parallel polysomnography (CPAP titration). The control group was not subject to the treatment. The OSA group was re-examined after 3-month CPAP treatment (Visit 2). The study protocol is presented in Fig. 1. A 12-channel polysomnography was conducted according to the American Academy of Sleep Medicine (AASM) guidelines (AASM 2005), using a SOMNOlab 2 device (Weinmann Emergency Medical Technology, Hamburg, Germany). The following variables were used in the final analysis: number of apneas and hypopneas per hour of sleep, apnea/hypopnea index (AHI), average and minimum arterial oxygen saturation, and overall desaturation index, i.e., the number of desaturations per hour of sleep (ODI). The ESS was used to assess daily sleepiness. Therapeutic CPAP pressure (i.e., the minimum pressure value generated by the device to maintain unobstructed airway) was defined in the process of CPAP titration with the use of an auto-CPAP device (REMStar; Philips Respironics, Murrysville, PA) under polysomnographic control. The effective duration of CPAP home therapy was assessed on the basis of data collected from CPAP device memory cards and analyzed with EncorePRO software (Philips Respironics, Murrysville, PA).

Table 1 Baseline characteristics of the obstructive sleep apnea (OSA) and control patients

Parameter		Controls	OSA patients
Age (years)		55.2 ± 2.7	54.4 ± 3.0
BMI (kg/m^2)		31.7 ± 1.2	33.9 ± 2.4∗
Gender	Male, *n* (%)	10 (66.7)	29 (74.4)
	Female, *n* (%)	5 (33.3)	10 (25.6)
Hypertension, n (%)		11 (73.3)	35 (89.7)

Data are means ±SD and number (%) of subjects. *BMI* body mass index; ∗$p < 0.001$ between the two groups

2.2 Evaluation of Heart Rate Variability (HRV)

HRV evaluation was made on the basis of ECG wave obtained from a full 24-h Holter recording of high quality, performed with a 3-channel monitor (Aspel; HolCARD 24 W, Zabierzów, Poland), according to the clinical practice guidelines of the European Society of Cardiology (ESC 1996). The following parameters were analyzed in the time-domain: SDNN, SDANN in consecutive 5-min intervals, and RMSSD. The spectral power of HRV was analyzed in the following frequency bands: ULF, VLF, LF, and HF.

2.3 Statistical Elaboration

Data were expressed as means ±SD or medians with minimum-maximum values. Since the intragroup results had a skewed distribution, the Mann-Whitney U test was used for comparisons. Comparisons between the study and control groups were made with a *t*-test for quantitative variables and a Chi-squared test for qualitative variable. The analysis of changes between visits in the OSA group was done with the Wilcoxon signed-rank test for repeated measurements. The

Table 2 Inclusion and exclusion criteria, comorbilities and pharmacotherapy

Inclusion criteria
Age > 18 years
Severe OSA (AHI ≥ 30/h)
ESS score > 10 points
Confirmed CPAP efficacy (AHI <10/h)
Exclusion criteria
Age < 18 years
AHI < 30/h
Low-quality ECG Holter
Comorbidities
Central/mixed sleep apnea
Heart failure
Ischemic heart disease
History of myocardial infarction
Persistent atrial fibrillation
History of cardioversion or ablation
History of stroke
Renal insufficiency
Liver insufficiency
Diabetes
Neoplasm
Pharmacotherapy
Beta-blockers
Calcium blockers
Digoxin, ivabradine

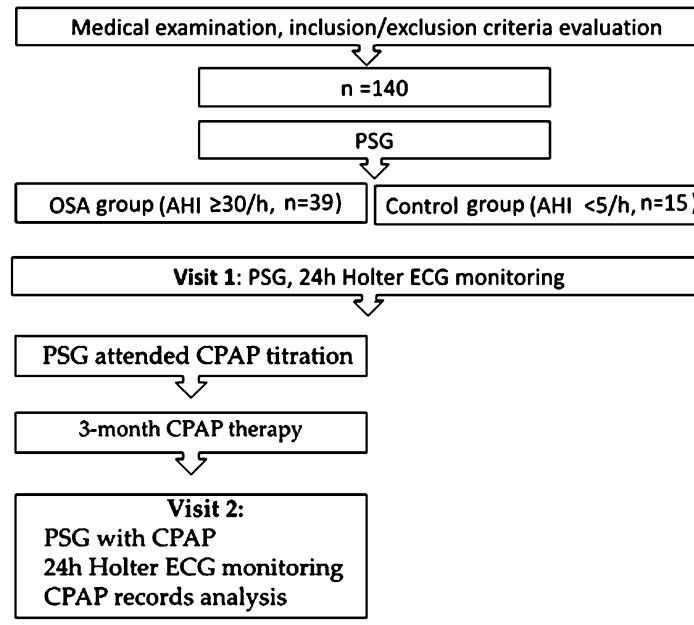

Fig. 1 Scheme of study paradigm

Table 3 Heart rate variability parameters in controls and in obstructive sleep apnea (OSA) patients at baseline (Visit 1) and in OSA patients after 3-month CPAP treatment (Visit 2)

	Controls	OSA patients	
Parameter	Visit 1		Visit 2
ESS (points)	5 (0–8)	15 (11–18) ∗	5 (1–9) ǂ
AHI (episodes per hour)	2.5 (0–4.7)	51 (31–85) ∗	4 (0–8) ǂ!
ODI (episodes per hour)	2 (0–3.6)	49 (25–87) ∗	26 (0–5.2) ǂ!
Average nocturnal SaO_2 (%)	96 (94–97)	91 (81–95) ∗	95 (94–97) ǂ
Min. SaO_2 (%)	90 (87–92)	75 (50–86) ∗	87 (83–90) ǂ !!
SDNN (ms)	138 (115–161)	114 (76–166) †	143 (83–176) ǂ
SDANN (ms)	124 (110–141)	99 (53–132) ∗	123 (70–212) ǂ
RMSSD (ms)	32 (29–37)	32 (23–37)	37 (25–43) ǂ !!
ULF (ms^2)	119 (115–124)	141 (128–176) ∗	128 (112–160) ǂ !!
VLF (ms^2)	519 (507–531)	631 (553–773) ∗	561 (516–694) ǂ !!
LF (ms^2)	416 (231–431)	476 (435–565) ∗	439 (401–497) ǂ !!
HF (ms^2)	351 (339–361)	311 (286–339) ∗	329 (245–391) ǂ !!
TP (ms^2)	1,405 (1,214–1,431)	1,557 (1,453–1,776) ∗	1,472 (1,325–1,626) ǂ !!
LF/HF	1.18 (0.66–1.24)	1.55 (1.29–1.78) ∗	1.37 (1.09–1.73) ǂ !!

Data are means ±SD, medians (min-max), and number of episodes
ESS Epworth sleepiness scale, *AHI* apnea/hypopnea index, *ODI* desaturations per hour of sleep, *SaO_2* arterial oxygen saturation, *SDNN* standard deviation of NN intervals, *SDANN* standard deviation of the average NN intervals, *RMSSD* square root of the mean squared successive differences of NN intervals, *ULF* ultralow frequency < 0.0033 Hz, *VLF* very low frequency 0.003–0.040 Hz, *LF* low frequency 0.04–0.15, *HF* high frequency 0.15–0.40 Hz, *TP* total spectral power ∗ $p < 0.001$ and † $p = 0.003$ between Visit 1 patients vs. control subjects; ǂ $p < 0.001$ between Visit 2 vs. Visit 1 same patients; ! $p < 0.05$ and !! $p < 0.001$ between Visit 2 patients vs. controls

associations among variables were assessed with a multivariate analysis of linear regression. The statistical significance level was set at $\alpha = 0.05$. The analyses were conducted with the R-3.0.2 project for statistical computing (R Foundation for Statistical Computing; Vienna, Austria).

3 Results

The SDNN, SDANN, and HF spectral power values were significantly lower in the OSA group. On the other side, ULF, VLF, LF, TP, and the LF/HF ratio of spectral power values were all significantly greater in the OSA patients than those in the control subjects. The number of ODI was manifold greater in the OSA patients than in controls (Table 3). The increases in ODI in the OSA patients were associated with significantly higher VLF, LF, TP, and the LF/HF spectral power ratio (Table 4). The evaluation of these associations was controlled for age, gender, body mass index (BMI), systolic (SBP) and diastolic (DBP) blood pressure, and a history of hypertension. There were no other associations among the OSA characteristics and frequency- or time-domain spectral data.

Three-month CPAP treatment resulted in significant increases in SDNN, SDANNN, RMSSD, and the HF spectral power, while the ULF, VLF, LF, TP bands, and the LF/HF ratio all decreased in OSA patients. The increases in SDNN and SDANN after treatment reached the level present in controls. On the other side, ULF, VLF, LF, TP bands, and the LF/HF, despite the decreases, remained at a level significantly higher than those in controls (Table 3).

4 Discussion

OSA patients manifest ANS dysfunction resulting from the pathophysiological events related to recurring episodes of apnea, hypoxia, and awakenings (Stein and Pu 2012; Somers et al. 1995). The ANS dysfunction is present not only during sleep but also during daytime (Narkiewicz and Somers 2001; Carlson et al. 1996). The ANS

Table 4 Associations between the increase in the number of desaturations per hour of sleep (ODI) and the spectral power frequency bands of heart rate variability in OSA patients

ODI	*r*	SE	*p*
VLF (ms^2)	2.074	0.953	0.038
LF (ms^2)	1.551	0.584	0.013
TP (ms^2)	3.629	1.272	0.008
LF/HF	0.007	0.003	0.018

r regression coefficient, *SE* standard error, *VLF* very low frequency 0.003–0.040 Hz, *LF* low frequency 0.04–0.15, *TP* total spectral power

dysfunction in OSA not only is a syndrome's manifestation but is at play in the development of cardiovascular complications. Attempts have been made to define OSA-related HRV changes, assessed by way of ECG Holter monitoring, as a screening tool. Although promising, the results of those studies have not yet defined the exact role of ECG Holter monitoring in the diagnostics of OSA (Gong et al. 2016; Harrington et al. 2013; Hayano et al. 2013; Sun et al. 2011; Roche et al. 1999b). Therefore, the goal of the present study was to reevaluate HRV changes in a group of fairly homogenous OSA patients in terms of disease severity, comorbidities, and therapy. We found significantly lower values of SDNN and SDANN in severe OSA, compared to control subjects. These results point to a lower parasympathetic activity, which conforms with a shift in ANS balance toward the sympathetic side. Our results are in line with those of Narkiewicz and Somers (2001), who have shown increased muscle sympathetic nerve activity in OSA, both during sleep and wakefulness. Those authors suggested that increased sympathetic tone could be related to altered baroreceptor reflex, which has been further confirmed by Carlson et al. (1996). In the present study, patients with the highest AHI had the lowest SDNN, the corollary of which is patients with severe OSA had a low level of advantageous, particularly from the standpoint of cardiovascular function, parasympathetic activity. Along the same line of evidence, Aydin et al. (2004) and Véber et al. (2014) have shown a significant decrease in time-domain of HVR in OSA. On the other hand, Karasulu et al. (2012) have reported the opposite tendency in a study in 30 OSA patients of differing disease severity and comorbidities, which in addition was not based on 24-h ECG recordings. There are also studies that fail to show the presence of any appreciable differences in the time-domain of HRV between OSA patients and healthy subjects (Lado et al. 2012; Zhu et al. 2012). In this regard, it is worth noting that in the present study, the medians of time-domain parameters in severe OSA, although significantly lower than those in non-OSA controls, still met the limit of normal values suggested in the guidelines (ESC 1996). Thus, we presume that the usefulness of time-domain analysis of HRV in the diagnostics of severe OSA remains unsettled.

In this study, we also showed that patients with severe OSA had a significant decrease in HF spectral power with simultaneous increases in LF, VLF, ULF, and the overall TP value. A low HF, compared to control subjects, points to a decrease in parasympathetic activity, the reasoning further confirmed by an increase in the LF/HF ratio. A counterincrease in sympathetic activity might thus be presumed. We further showed that an increase in ODI was related to the increases in LF spectral power and LF/HF ratio. Hypoxia, expressed in the ODI values, may add to the stimulation of sympathetic activity through the chemoreceptor reflex (Narkiewicz and Somers 2001; Somers et al. 1995), and it increases the VLF spectral power, which we also noticed. The VLF spectrum presumably represents the exaggerated heart rate oscillations, caused by recurring episodes of apnea during sleep, in a short time span of 1–5 min. An increase in VLF, noticed also in other studies (Karasulu et al. 2012; Aydin et al. 2004; Gula et al. 2003), has been proposed a marker of OSA (Malik and Camm 1993).

Palma et al. (2014) have described two basic phenotypes of OSA syndrome: with and without hypoxia, examining a group of 129 patients with severe OSA. The hypoxic phenotype is characterized by an increase in sympathetic activity, expressed as increases in both LF spectral power and LF/HF ratio, compared to the non-hypoxic OSA or healthy subjects. The presence of two OSA phenotypes, distinguished by the severity of hypoxia and relating the ANS

disorders to the hypoxic phenotype, but having a similar AHI, is an interesting clinical concept as it would enable a prompt association of cardiovascular risk with hypoxia. However, the use of frequency-domain of HRV as a diagnostic tool for OSA also is limited by a wide range of normal spectral power values for the respective frequencies. Nonetheless, the present study showed that increased spectral power of LF and VF, with a concurrent fall in HF, would point to severe OSA. Likewise, the LF/HF ratio exceeding 2.5 might suggest increased sympathetic activity, which could suggest the diagnosis of OSA. Changes in the frequency-domain of HRV we noticed in this study are in line with the notion that hypoxia is a key factor linking severe OSA with altered ANS activity.

We also showed that CPAP treatment led to distinct increases in all time-domain parameters of HRV. Concerning the frequency-domain, HF spectral power increased, with concurrent decreases in ULF, VLF, LF, and LF/HF ratio. A significant decrease in TP also was noticed. Such changes may be considered as reflecting a clinically favorable shift back to parasympathetic edge over sympathetic modulation as a result of CPAP treatment. There are rather scarce studies evaluating the influence of CPAP on HRV. Roche et al. (1999a) have evaluated the effects of 3-month CPAP treatment on the time- and frequency-domains of HRV in severe OSA (14 patients; average AHI = 50.6/h), based on a 24-h ECG recordings. The authors notice an increasing tendency in the time-domain parameters, and distinct increases in HF, with concurrent decreases in LF and VLF spectral power. Chrysostomakis et al. (2006) have evaluated the effects of 2-month CPAP treatment on the time-domain of HVR in 31 patients with moderate and severe OSA. The authors also notice an increasing tendency for SDNN and SDANN. Limphanudom et al. (2007) have evaluated the effects of 6-month CPAP treatment on the time- and frequency-domains of HRV in a small group of ten patients with severe OSA. These authors failed to notice any appreciable differences before and after therapy.

In summary, in comparison to healthy subjects, patients with severe OSA have decreases in time-domains and increases in frequency-domains of HRV which are strongly suggestive of a disadvantageous, from the clinical standpoint, shift in the balance of parasympathetic/sympathetic activity toward the latter. CPAP treatment significantly, albeit not entirely, reversed these changes. These findings show that HVR analysis, based on an easily accessible 24-h ECG Holter monitoring, may help identify the presence of OSA and the effectiveness of CPAP treatment, which makes it a potentially valuable clinical tool. Nonetheless, the usefulness of the HRV evaluation is limited by all too often comorbidities accompanying OSA and by their pharmacotherapy, which may affect the HVR.

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

Ethical Approval The project was approved by the Bioethics Committee of the Jagiellonian University in Cracow, Poland (permit KBET/115/B/2010). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Subterranean Pulmonary Rehabilitation in Chronic Obstructive Pulmonary Disease

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Abstract

Pulmonary rehabilitation (PR) has been recommended as an integral part of treatment for patients with chronic obstructive pulmonary disease (COPD). Climate therapy in salt mine chambers has been found of benefit in chronic respiratory diseases. The study compares long-term effects of underground PR in the Wieliczka Salt Mine with that conducted on the surface. There were 42 COPD patients enrolled in the study, with FEV_1/FVC <0.7 predicted and post-bronchodilator reversibility <12%, randomized into pulmonary rehabilitation in the mine (Group I, n = 23) and PR on the surface (Group II, n = 19). The outcomes consisted of lung function variables, exercise performance (6-min walk test – 6MWT), dyspnea (mMRC), and compliance with the disease and quality of life (COPD Assessment Test – CAT) and BODE index, compared at baseline (P0), end (P1), and 6 months after pulmonary rehabilitation (P2). The findings were that subterranean pulmonary rehabilitation significantly reduced CAT score ($p < 0.001$), BODE index ($p = 0.004$), and dyspnea (mMRC) ($p = 0.001$) and increased distance in 6MWT ($p < 0.001$), compared with its equivalent conducted on the surface. Further, beneficial effect of subterranean treatment was sustained during the following half a year as opposed to the effect noticed on patients treated on the surface. We conclude that subterranean pulmonary rehabilitative treatment reduces symptoms and improves exercise tolerance to a greater and sustained extent, compared to a similar treatment on the surface, in patients suffering from COPD.

Keywords

COPD · Pulmonary rehabilitation · Speleotherapy · Subterranean therapy

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1 Introduction

Chronic obstructive pulmonary disease (COPD) is one of the most common causes of hospitalization and disability (Gibson et al. 2013). A chronic and progressive nature of COPD and periodic exacerbations significantly deteriorate patients'

quality of life and contribute to a poor prognosis. The most common symptoms reported by patients with COPD are dyspnea and cough. Reduced physical activity occurs as a result of chronic breathlessness and fatigue. The subsequent deconditioning is aggravated by systemic effects such as peripheral muscle, cardiac, nutritional, and psychosocial dysfunction. As a consequence, quality of life significantly deteriorates. Self-management problems caused by dyspnea lead to a sense of social isolation, depressed mood, and anxiety (Reardon et al. 2005; Troosters et al. 2005). The most important goals of COPD treatment consist of reductions in symptom intensity and improvement in exercise tolerance, combined with slower disease progression and fewer exacerbations (GOLD 2019). Pulmonary rehabilitation has become a standard treatment for COPD patients due to its effectiveness in reducing somatic and psychosocial symptoms and consequently reducing hospitalization and treatment costs (Ries et al. 2007; Nici et al. 2006).

Because air pollutants, such as organic and inorganic dust and gases, influence symptomatology and progression of COPD (van der Molen et al. 2018), it has been suggested that pulmonary rehabilitation programs should be held in locations where these conditions do not occur. These locations are understandably underground health resorts and salt mine chambers. A specific method of climate therapy is subterraneotherapy or speleotherapy, i.e., conducting rehabilitation programs in an underground environment (Nurov 2010). The main therapeutic factors in subterraneotherapy are the air quality and stability of climatic conditions, which includes a high concentration of minerals, high relative humidity and ionization, and very low level of dust, pollution, and bioaerosol (Beamon et al. 2001). Several studies indicate that subterraneotherapy is an effective and supportive method of treatment of chronic airway diseases (Kostrzon et al. 2015b; Horvath 1986).

The Wieliczka Salt Mine is a World Heritage site, located in southern Poland. It consists of a network of nearly 300 km of subterranean tunnels. Salt mining began there at the beginning of the thirteenth century. The mining was practically phased out in 1996. The site has been turned into a tourist facility since. An allergy sanatorium has been established in a few salt chambers in 1964; turned into a rehabilitation facility later on. In 2011, the Wieliczka Salt Mine achieved the status of an underground health resort, recently focusing on pulmonary and allergy therapies. The complex of four salt chambers of the resort is located at depth of 135 m underground. The chambers are equipped with training equipment, fitness tools, inhalators, and are furnished to relax. The microclimate of the salt chambers is characterized by a low, stable temperature of 13–14.5 °C, high relative humidity of 60–75%, high concentration of salt aerosol of 2.7–8.1 mg/m^3, ionization of 1200–4700 $aeroions/cm^3$, and almost lack of allergens and particulate matter (Table 1). The air is free from harmful radiation, pollution, or toxic agents (Rogula-Kozłowska et al. 2017; Kostrzon et al. 2015a; Wiszniewski 2015).

The aim of this study was to evaluate the additive influence of the microclimate of the salt mine chambers on the effectiveness of pulmonary rehabilitation in patients with stable COPD.

2 Methods

2.1 Patient Recruitment and Study Design

This randomized control study was conducted from May 2015 to August 2016 in the underground Wieliczka Salt Mine Health Resort. Originally, there were 55 consecutive, stable COPD patients recruited for the study. Forty nine patients qualified to participate in the project. The remaining five patients did not meet inclusion criteria, and one patient was excluded due to advanced cardiovascular insufficiency, which is a contraindication for pulmonary rehabilitation. A full 3-week-long program of pulmonary rehabilitation was completed by 42 out of the 49 patients.

The main inclusion criterion was diagnosis of COPD based on the GOLD recommendations

Table 1 Climatic conditions in the underground salt chambers of the Wieliczka Salt Mine Health Resort

Parameter	Range
Temperature	12.9–14.5 °C
Relative humidity	59.9–74.8%
Ionization	1200–4700 aeroions/cm^3
Concentration of minerals in the air	2.7–8.1 mg/m^3
Total PM concentration	30 $\mu g/m^3$
Respirable PM concentration	7 $\mu g/m^3$

PM particulate matter (Rogula-Kozłowska et al. 2017; Kostrzon et al. 2015a; Wiszniewski 2015)

(GOLD 2019), most notably, spirometry demonstrating forced expiratory volume in 1 s to forced vital capacity (FEV_1/FVC) ratio of <0.7 after administration of 400 mg salbutamol, with post-bronchodilator FEV_1 reversibility of <12%. Patients with other acute or chronic airway diseases, cardiovascular diseases, or other health conditions that could exclude them from physical exercises or testing procedures were excluded from the study.

Patients were randomly assigned to Group I (n = 25; pulmonary rehabilitation in the salt chambers underground) and Group II (n = 24; pulmonary rehabilitation on the surface). The random assignment was carried out by a person who registered the patients and who was not responsible for performing or evaluating the study. The teams of physiotherapists, either working underground or on the surface remained blinded to the study purpose. The characteristics of the 42 patients who completed the study are presented in Table 2.

2.2 Pulmonary Rehabilitation Program

Pulmonary rehabilitation was, in general, based on the guidelines of the American Association of Cardiovascular and Pulmonary Rehabilitation (AACVPR) (Ries et al. 2007). Before rehabilitation commencement, a team of physiotherapists had a training course aimed at harmonizing the method. Next, patients' functional status was evaluated according to the study protocol. In both groups of patients, the program lasted for 3 weeks, five sessions of supervised training *per* week of 120 min each. The program was implemented according to the following scheme of classes:

- Endurance training on a cycloergometer and elliptical bike – 35 min
- Strength training of upper and lower limbs – 30 min
- General fitness exercises, combined with breathing exercises – 55 min
- Health education – 30 min (two times a week)

Each training workout started with a short 5-min warm-up. There was a 20–30-min break between individual classes.

The underground pulmonary rehabilitation was performed in a complex of salt chambers in the Wieliczka Salt Mine at a depth of 135 m. Climatic conditions in the underground rehabilitation chambers are presented in Table 1. The rehabilitation on the surface was carried out in a gym of the Wieliczka Salt Mine Health Resort.

2.3 Outcome Measures

The outcomes consisted of the effects of a 3-week-long outpatient pulmonary rehabilitation on lung function, exercise performance (6-min walking distance – 6MWD), disability due to dyspnea (modified Medical Research Council scale – mMRC), on patients' quality of life (COPD Assessment Test – CAT), and a composite measure of prognosis (BODE index). These

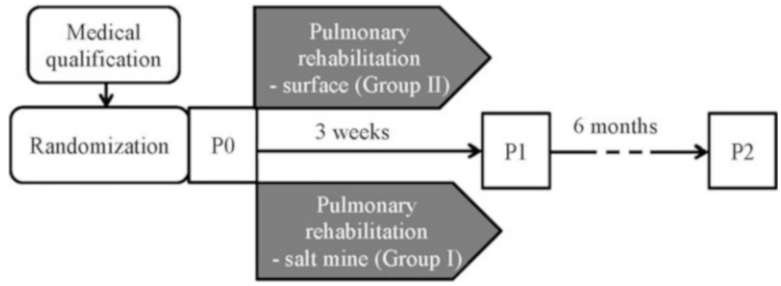

Fig. 1 Schematic of study paradigm: *P0*, baseline level; *P1*, end of pulmonary rehabilitation; and *P2*, follow-up after 6 months

parameters were assessed and statistically elaborated three times: baseline (P0), end of pulmonary rehabilitation (P1), and at 6 months' follow-up (P2). The diagram of the study procedure is shown in Fig. 1.

2.4 Pulmonary Function, Exercise Performance, Dyspnea, and Quality of Life

Spirometry was performed with the use of Lungtest SB spirometer (MES; Skawina, Poland) calibrated daily as recommended by the ATS guidelines (Miller et al. 2005). A reversibility test was then performed after administration of 400 mg salbutamol. The FEV_1 and FEV_1%VC values were compared with the reference values (Sterk et al. 1993). Resting arterial blood oxygen saturation was assessed with an Oxytest 2000 M/B finger pulse oximeter (MES; Skawina, Poland).

Exercise performance was assessed using the 6MWT, performed according to the ATS protocol (ATS 2002). The test was performed in a 30-m-long hallway. Oxygen saturation, heart rate, and arterial blood pressure were monitored before and after 6MWT. A modified 0–10 Borg scale was used to estimate dyspnea before and after the test. Disability due to the intensity of dyspnea was assessed on a 5-level mMRC (Bestall et al. 1999).

CAT test was used to assess the severity of COPD symptoms, the impact of the disease on daily life, and general physical and mental state of patients) (Jones et al. 2009). A score of >10 points was considered a threshold above which COPD symptoms were considered severe (GOLD 2019).

BODE index was used as a prognostic indicator. The index takes into account the systemic nature of the disease and is calculated on the basis of body mass index (B), bronchial obstruction (O), dyspnea (D), and exercise tolerance (E) of patients. The index score ranges from 0 (lowest risk) to 10 (highest risk) (Celli et al. 2004).

2.5 Statistical Elaboration

Data were reported as means ±SD. The initial differences between Group I and Group II were compared with one-way analysis of variance (ANOVA) for quantitative data and a χ^2 test for qualitative data. Differences among the baseline, post-program, and 6 months' follow-up values, including the differences between the two groups, were compared with two-way analysis of variance for repeated measures, taking into account the measured value as one variable and the place where rehabilitation took place as the other variable. If the sphericity assumption was unmet, the degrees of freedom were calculated with the Greenhouse-Geisser correction. A p-value of <0.05 defined statistically significant differences. The analysis was performed using a commercial IBM SPSS v21 Statistics (IBM Corp; Armonk, NY).

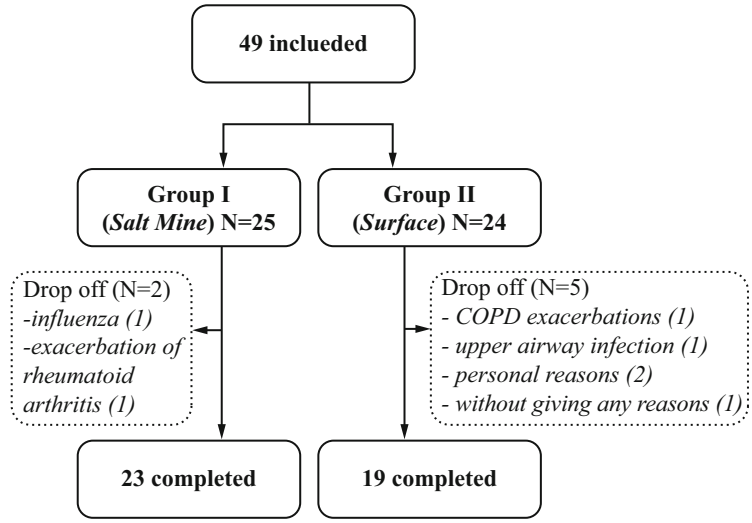

Fig. 2 Study flow diagram

3 Results

Two out of the 25 patients in Group I (pulmonary rehabilitation performed in the salt chambers underground) failed to complete the rehabilitation program for medical reasons, 1 patient due to influenza and another due to an exacerbation of rheumatoid arthritis. Two out of the 24 patients in Group II (pulmonary rehabilitation performed on the surface) discontinued rehabilitation for medical reasons, 1 patient due to COPD exacerbation and another due to upper airway infection requiring antibiotic therapy. Three other patients in this group also failed to complete the program due to personal reasons (Fig. 2). Patients were judged to fulfill the rehabilitation when they missed no more than 2 out of the 15 sessions scheduled. The baseline characteristics of the two groups were presented in Table 2. No statistically significant differences were detected between the two groups.

3.1 Pulmonary Rehabilitation and Respiratory Function

In comparison to baseline (P0), FEV_1 and FEV_1% VC did not differ significantly after the completion of rehabilitation (P1) and after 6 months' follow-up (P2) in either group (Table 3). However, the dynamics of FEV_1 change were significantly different between the two groups ($p = 0.034$). FEV_1 increased by 4.3% in Group I and it decreased by 3.0% in Group II at P2 (Fig. 3). The mean SpO_2 remained inappreciably different between the two groups at the three measurement points. There was a significant increase in SpO_2, of similar dynamics in both pulmonary rehabilitation groups, from 93.7 ± 2.3% at P0 to 94.6 ± 2.1% at P2 ($p = 0.011$).

3.2 Pulmonary Rehabilitation and Exercise Performance

After the completion of pulmonary rehabilitation, the distance covered by patients in 6MWT significantly increased from Point 0 to Point 1 in both groups (Fig. 4). In Group I, the increase was from 489.0 ± 88.5 m to 562.5 ± 69.7 m, respectively, followed by a decrease to 529.6 ± 84.0 m at P2. In group II, the increase was from 487.3 ± 68.9 m to 529.7 ± 71.7 m, respectively, followed by a decrease to 490.1 ± 67.0 m at P2 (Table 3). The improvement in exercise tolerance at Point 1 compared to the baseline level was significant in both groups ($p < 0.001$). At Point 2, the 6MWD

Table 2 Baseline characteristics of the pulmonary rehabilitation patients

Variables	Group I salt mine (n = 23)	Group II surface (n = 19)
Gender (M/F) (n)	15/8	9/10
Airflow limitation (mild/moderate/severe/very severe) (n)	2/15/5/1	2/11/5/1
COPD group (A/B/C/D) (n)	0/15/0/8	1/8/0/10
Smoking status (non-smoker/former smoker/active smoker) (n)	1/11/10	0/14/5
Age (years)	62.1 ± 8.3	65.2 ± 6.0
BMI (kg/m^2)	26.9 ± 2.8	27.2 ± 6.6
Pre-bronchodilator FEV_1 (% predicted)	57.7 ± 16.5	54.1 ± 19.8
Post-bronchodilator FEV_1 (% predicted)	60.7 ± 16.1	55.6 ± 16.8
FEV_1%VC (%)	49.9 ± 10.3	44.0 ± 13.7
SpO_2 (%)	93.7 ± 2.8	93.7 ± 1.8
HR (beats/min)	76.0 ± 12.9	83.9 ± 14.8
6MWD (m)	489.0 ± 88.5	487.3 ± 68.9
mMRC (score)	1.73 ± 0.77	1.74 ± 0.73
CAT (score)	19.3 ± 5.3	20.5 ± 7.2
BODE index	1.71 ± 1.21	2.36 ± 1.86

COPD chronic obstructive pulmonary disease, *BMI* body mass index, *FEV_1* forced expiratory volume in 1 s, *VC* vital capacity, *SpO_2* peripheral arterial oxygen saturation, *HR* heart rate, *6MWD* 6-min walking distance, *mMRC* modified Medical Research Council scale, *CAT* COPD Assessment Test. There were no significant inter-group differences in none of the variables

distance remained significantly longer than that at baseline at Point 2 in Group I ($p = 0.001$), but not so in Group II ($p > 0.05$).

3.3 Pulmonary Rehabilitation and Dyspnea Intensity, COPD Symptoms, and Quality of Life

After both underground and surface pulmonary rehabilitation, dyspnea intensity significantly decreased, compared to baseline, as assessed by mMRC ($p = 0.001$) (Table 3). However, dyspnea decreased significantly more in Group I (underground) than in Group II (surface) at both Point 1 and Point 2 ($p = 0.020$) (Fig. 5). A decrease in dyspnea was sustained at Point 2 in both groups ($p = 0.050$).

In both underground and surface pulmonary rehabilitation groups, a similar reduction in the intensity of COPD symptoms, as assessed by the CAT, was noted at P1, compared to baseline ($p < 0.001$). After 6 months' follow-up (P2), the intensity of COPD symptoms remained lower, and it did not differ from that at P1 in both groups (Table 3). Likewise, there was no inter-group difference in the dynamics of a decrease in symptom intensity (Fig. 6).

The BODE index in the underground pulmonary rehabilitation group was somehow lower at baseline than that in the surface rehabilitation. Nonetheless, BODE index decreased significantly after the completion of rehabilitation and remained at a lower level after 6 months' follow-up in both groups. Differences in comparison to the initial values were significant (P1 vs. P0 ($p = 0.004$) P2 vs. P0 ($p = 0.005$)), whereas the P1 vs. P2 difference was insignificant (Table 3). The dynamics of BODE index changes were akin to each other in the two groups (Fig. 7).

4 Discussion

The use of subterranean environments, speleotherapy, is a therapeutic measure in the treatment of COPD. It is widely applied in some Central and Eastern European countries (Beamon et al. 2001). Experimental in vitro studies indicate that speleotherapy induces changes in morphology and protein expression of lung and skin fibroblasts, which may lay ground for therapeutic

Table 3 Lung function, physical performance, and symptoms after underground in salt mine chambers (Group I) surface (Group II) pulmonary rehabilitation

	Baseline (Point 0)		End of rehabilitation (Point 1)		6 months' follow-up (Point 2)		p		
Variables	Group I	Group II	Group I	Group II	Group I	Group II	Place of rehabilitation	Time point of assessment	Place x point
FEV_1 (% predicted)	57.7 ± 16,5	54.1 ± 19.8	57.2 ± 15.6	55.1 ± 19.7	61.5 ± 14.8	52.0 ± 16.8	0.418	0.840	0.034
FEV_1%VC (%)	49.9 ± 10.3	44.0 ± 13.7	47.4 ± 11.4	43.1 ± 13.2	49.4 ± 9.8	42.0 ± 12.5	0.174	0.436	0.517
SpO_2 (%)	93.7 ± 2.8	93.7 ± 1.8	–	–	94.6 ± 2.2	94.6 ± 2.0	0.992	0.011	0.915
6MWD (m)	489.0 ± 88.5	487.3 ± 68.9	562.5 ± 69.7	529.7 ± 71.7	529.6 ± 84.0	490.1 ± 67.0	0.286	<0.001	0.031
mMRC (score)	1.7 ± 0.8	1.7 ± 0.7	1.0 ± 0.5	1.5 ± 0.6	1.2 ± 0.8	1.6 ± 0.7	0.020	0.003	0.172
CAT (score)	19.3 ± 5.3	20.5 ± 7.2	9.0 ± 3.8	12.6 ± 6.5	10.8 ± 4.8	12.7 ± 7.5	0.153	<0.001	0.373
BODE index	1.7 ± 1.2	2.4 ± 1.9	0.9 ± 0.9	2.1 ± 1.4	0.9 ± 1.0	2.1 ± 1.5	0.028	0.001	0.187

Data are means ±SD

FEV_1 forced expiratory volume in 1 s, *FVC* forced vital capacity, *SpO_2* peripheral arterial oxygen saturation, *6MWD* 6-min walking distance, *mMRC* modified Medical Research Council scale, *CAT* COPD assessment test, *BODE index* Body mass index, airflow obstruction, dyspnea and exercise capacity

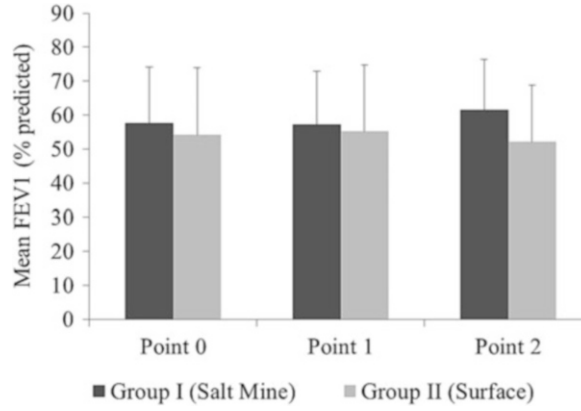

Fig. 3 Forced expired volume in 1 s (FEV_1), compared to baseline (Point 0), after completion of pulmonary rehabilitation (Point 1), and then after 6 months' follow-up (Point 2) depending on the rehabilitation site (underground salt mine chambers vs. surface). There were no statistically significant differences across the groups and measurements points

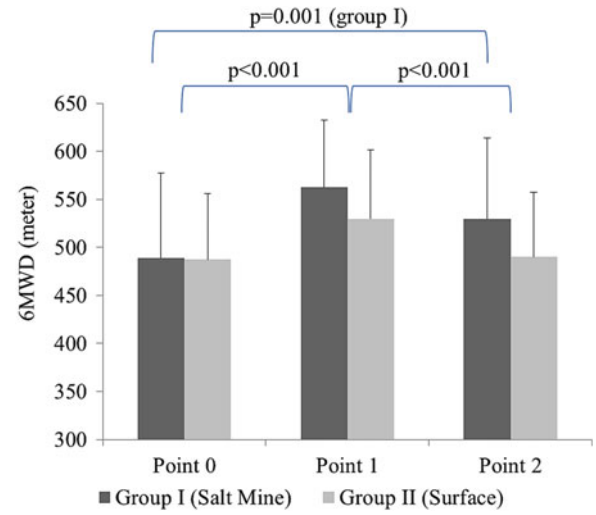

Fig. 4 Six-minute walk test (6MWT), compared to baseline (Point 0), after completion of pulmonary rehabilitation (Point 1), and then after 6 months' follow-up (Point 2) depending on the rehabilitation site (underground salt mine chambers – Group I vs. surface – Group II). The uppermost p-brackets pointing to the darker bars representing Group I indicate significant difference between baseline and follow-up measurement points in this group. The lower p-brackets pointing to the abutment of bars representing Group I and Group II denote significant difference in either group between the measurement points indicated

effects (Lăzărescu et al. 2014). Numerous studies point to the impact of air pollution on lung diseases, inter alia, on COPD, causing higher mortality, exacerbations, and intensification of symptoms (GOLD 2019; Guerreiro et al. 2018; Anto et al. 2001). The present study was designed to address a direct influence of underground climate, including pure air quality, on the effectiveness of rehabilitative physiotherapy in COPD patients. The unique characteristics of the microclimate within the salt caves include stable air temperature, moderate-to-high humidity, the presence of fine aerosol components, notably sodium chloride, and the lack of airborne pollutants and bioaerosol (Rogula-Kozłowska et al. 2017; Kostrzon et al. 2015a; Wiszniewski 2015). We found that pulmonary rehabilitation caused a significant improvement in exercise performance in both underground- and surface-treated groups of patients. However, 6MWD

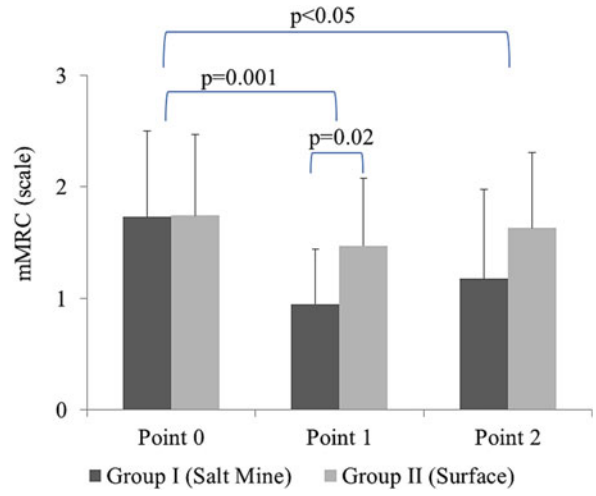

Fig. 5 Disability due to dyspnea, according to the modified Medical Research Council (mMRC) scale, compared to baseline (Point 0), after completion of pulmonary rehabilitation (Point 1), and then after 6 months' follow-up (Point 2) depending on the rehabilitation site (underground salt mine chambers – Group I vs. surface – Group II). The p-brackets pointing to the abutment of bars representing Group I and Group II denote significant difference in either group between the measurement points indicated. In addition, there was a significant difference between the two groups at Point 1

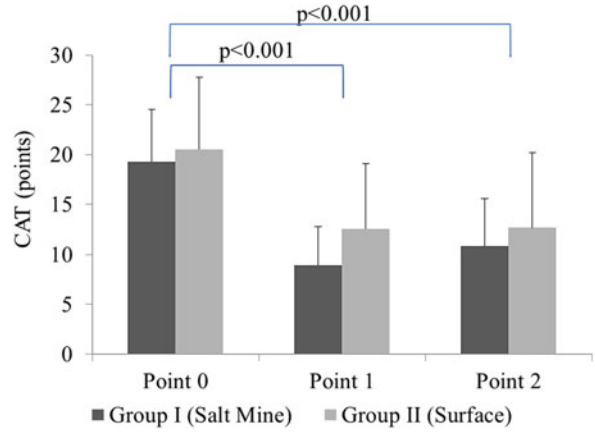

Fig. 6 Severity of COPD symptoms, compared with baseline (Point 0), after completion of pulmonary rehabilitation (Point 1), and then after 6 months' follow-up (Point 2) depending on the rehabilitation site (underground salt mine chambers – Group I vs. surface – Group II). The p-brackets pointing to the abutment of bars representing Group I and Group II denote significant difference in either group between the measurement points indicated

tended to increase to a greater degree in patients treated in the underground environment. The intervention in the salt underground chamber also had a long-term effect as physical improvement was sustained during the 6-month-long follow-up. Likewise, airflow obstruction, reflected in FEV_1 measurement, remained decreased in the underground-treated patients during the follow-up, whereas it showed an increasing trend in patients treated on the surface. Kendrová et al. (2016), in a study concerning the influence of speleotherapy on pulmonary rehabilitation in COPD patients, have reported a significant improvement in physical tolerance, quality of life (symptoms domain), and anxiety, with no significant changes noticed in the control group. Those effects, however, concerned the short term as opposed to the present study in which we followed the patients up to half a year after the completion of rehabilitation. The underground

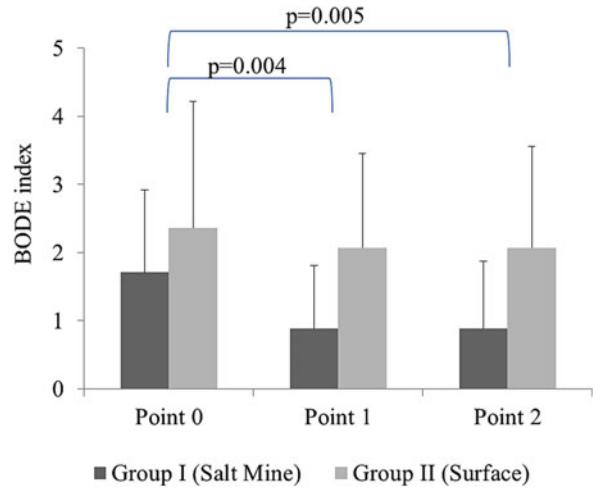

Fig. 7 BODE index, a composite of body mass (B), bronchial obstruction (O), dyspnea (D), and exercise tolerance (E), compared to baseline (Point 0), after completion of pulmonary rehabilitation (Point 1), and then after 6 months' follow-up (Point 2) depending on the rehabilitation site (underground salt mine chambers – Group I vs. surface – Group II). The p-brackets pointing to the abutment of bars representing Group I and Group II denote significant difference in either group between the measurement points indicated

rehabilitative environments also differed in both studies, with a cave in the former study and the salt mine chambers in the in present one.

A systematic review on the influence of halotherapy, consisting of the inhalation of micronized dry salt in a chamber, mimicking salt environment, on lung function mentions three case-control studies that report improved, to a variable degree, respiratory function. Most notably, improvements were noticed in FVC, FEV_1, arterial blood oxygen saturation, or arterial partial pressure of oxygen (Rashleigh et al. 2014). In the present study, despite the lack of spirometric changes at the end of rehabilitation, there was observable improvement in symptoms' perception, as assessed by the mMRC and CAT. Inhaled salt therapy may ameliorate COPD symptoms by increasing the airway mucous secretion, and consequently its expectoration. That could result in dyspnea reduction and enhanced physical exercise capacity. The latter effect was substantial in the patients treated in the salt mine chambers as they increase in 6MWD was almost doubled in comparison to the control group treated at the surface. That increase in 6MWD was more than twice the magnitude that defines the minimum clinically important difference (MCID) (Holland and Nici 2013), and it was still noticeable after half a year. It has been confirmed in previous studies that COPD perception, coping with disease, and quality of life are all interrelated (Tiemensma et al. 2016). Moreover the non-pharmacological adherence to therapy, depression, anxiety, and cognitive impairment shows significant relations with 6MWD (Pierobon et al. 2017). That suggest that being more fit and resilient increases patients' self-esteem and general mental state, which helps ameliorate symptoms, for instance, dyspnea perception, in COPD. Further, a patient with COPD, suffering from dyspnea and not having regular exercise routines, would most likely benefit from an exercise program tailored to his capability (Katajisto et al. 2012). In the present study, the level of patients' dyspnea was rather modest (the mean mMRC score of less than two), and the initial physical tolerance, assessed during 6MWT, indicated a rather good physical performance (the mean 6MWT of less than 450 m); however the patients had not been interviewed about their daily exercise routine.

As concluded in a Cochrane review, breathing exercises over 4–15 weeks improve functional exercise capacity in COPD patients compared to no intervention, but there are no consistent effects of exercise on dyspnea or health-related quality of

life (Holland et al. 2012). In the present study, therapeutic effect was significant after 3 weeks' treatment, but it needs to be considered that patients were offered 120 min of a complex therapeutic intervention every day. The influence of breathing exercises in COPD patients may vary according to underlying pathophysiological mechanisms, the technique employed, and the conditions of training. Nonetheless, it appears that the salt mine's conditions help achieve a long-standing therapeutic effect.

5 Summary and Conclusions

In the present study, pulmonary rehabilitation, in general, improved exercise tolerance, reduced symptoms, and has a positive influence on the COPD patient's quality of life and BODE index. A reduction in dyspnea was greater in those patients who underwent rehabilitation in the underground salt chamber environment. Six months after rehabilitation, exercise performance improved to a greater extent in patients treated in the salt mine chambers as opposed to that in patients treated in the standard conditions at the surface.

As the nature-related specifics of underground environments are highly variable, speleotherapy should not be considered as a single distinct therapy but rather a class of interventions (Lăzărescu et al. 2014). Results obtained with a specific treatment schedule in a defined cave or mine cannot be extrapolated to speleotherapy in general. Therefore, positive influence on the course of COPD of the rehabilitative program performed in the underground salt mine chambers noticed in the present study requires reconsideration in different environmental conditions, with relation to temperature, humidity, allergen presence, and other geophysical properties. Another limitation of this study is a relatively small sample size, outpatient setting, and a rather low expression of COPD symptoms, pointing to a mild disease course. Nonetheless, we believe we have shown that speleotherapy in the specific environment of underground salt chambers helps mitigate COPD symptoms and improves patients' physical capability. The therapy may, therefore, be recommended as a complementary treatment of COPD.

Conflicts of Interest MK, MS, and DA are employees of the Wieliczka Salt Mine. The other authors declare no conflicts of interest related to this article.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Regional Medical Chamber in Cracow, Poland (No. 38/KBL/OIL/2015 dated 15th of April 2015).

Informed Consent Informed written consent was obtained from all individual participants included in the study.

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Epidemiological Aspects of Low Back Pain

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Abstract

Low back pain (LBP) is a major health problem, particularly in the contemporary societies of highly developed countries. This study seeks to define the influence of basic demographic and social factors, such as gender, body mass, physical activity, and the type of work, on the occurrence of lumbosacral spine pain in the early and middle-late adulthood. The study was based on a self-reported survey, using the revised Oswestry Low Back Pain Disability Questionnaire to evaluate pain symptoms, and managing everyday tasks. Physical activity was evaluated on the Minnesota Leisure Time Physical Activity Questionnaire. We found that patients in the early adulthood had a significantly lower level of disability. The older patients had a greater low back pain and motion, sleeping, and social life problems. Neither did gender nor the type of work, leisure time physical activity, or body mass appreciably affect the level of disability due to low back pain in both younger and older patient groups. We conclude that, all else unchanged from the epidemiological standpoint, wear and tear of the spine structure naturally progressing with age seems a major determinant of the appearance of low back pain.

Keywords

Disability · Epidemiological aspects · Low back pain · Lumbosacral spine · Physical activity · Risk factors

1 Introduction

Low back pain is one of the biggest problems of modern medicine, leading to worrisome symptoms, work absenteeism, engagement of medical resources and services, and chronic disability in extreme cases. The etiology of low back pain is not full well clear. Further, there is a steady upward trend in its incidence (Allegri et al. 2016). A major issue is the lack of a precise knowledge about the risk factors for the occurrence of low back pain (Green et al. 2018). Many studies point to obesity as a risk factor, but most researchers

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underscore that this relationship is rather poorly documented (Heuch et al. 2013; Leboeuf-Yde 2000). Some other studies draw attention to more frequent back pain in patients with mental disorders (Chou et al. 2016; Cabak et al. 2015). Another risk factor might be the type of work performed, depending on whether it is white-collar or blue-collar work. Sedative lifestyle predisposes to spinal problems, particularly in office workers (Cabak et al. 2017; Curyło et al. 2017).

Gender may also potentially factor in the development of low back pain. Wáng (2015) has drawn attention to the period of menopause, which is associated with the acceleration of intervertebral disc dysfunction or injury, which increases the likelihood of developing spine pain syndromes. Women also are slower than men to recuperate from low back pain (Peul et al. 2008). The present study seeks to define the influence of basic demographic and social factors, such as gender, body mass index (BMI), physical activity, and the type of work on the occurrence of low back pain in the middle-aged adults.

2 Methods

The study was conducted from January to March 2017 in 80 patients (40 men and 40 women) in a rehabilitation outpatient clinic in Warsaw, Poland. Patients were evenly subdivided into two age groups: early (F/M – 15/19; mean age of 31.8 ± 5.2 years) and middle-late adulthood (F/M – 25/21; mean age of 59.2 ± 10.1 years). The revised Oswestry Low Back Pain Disability Scale (Fairbank and Pynsent 2000) was used to assess the intensity of pain symptoms. The scale consisted of ten items concerning the severity and changeability of pain; the endurance in sitting, standing, and walking; and the agility in lifting items, socializing, and traveling and also concerning personal self-care and sleep problems. Each item was evaluated on the 5-point Likert scale, where zero indicated no problems whatsoever and five indicated the most severe limitation of an activity as reported by the subject. Physical activity was evaluated on the Minnesota Leisure Time Physical Activity Questionnaire (MLTPAQ). The MLTPAQ assesses, in a subjective manner, physical activity of an individual when he is not engaged in daily work-associated activities (Richardson et al. 1994). The questionnaire consists of eight categories of physical activity, each having a different burden on the musculoskeletal system and thus a different energy expenditure. The quantification of results is in metabolic equivalents of task (MET), where 1 MET equals energy expenditure in terms of oxygen consumption per 1 kg of body mass while sitting at rest.

Data were expressed as means ±SD. Data distribution was checked with the Shapiro-Wilk test. Quantitative data were compared between the two age groups with a *t*-test or the Mann-Whitney test for normal and skewed distribution, respectively. Pearson's chi-squared test (χ^2) was applied to sets of categorical data to evaluate the probability of differences between them appearing by chance. Spearman's rank-order correlation was used to evaluate the correlation between the severity of low back pain and age, BMI, and physical activity. A p-value < 0.05 defined the statistically significant differences. A commercial Statistica v13 package was used for all analyses (StatSoft, Tulsa OK).

3 Results and Discussion

We found that the scores of low back pain intensity in early and middle-late adulthood periods, according to the Oswestry questionnaire, amounted to 0.89 and 2.78 points, respectively; the difference between the two groups was significant ($p < 0.001$). Likewise, pain instability and alterations in its intensity were significantly greater in middle-late adulthood ($p < 0.001$). All questionnaire's items related to musculoskeletal and motion agility scored more, i.e., demonstrated worse performance, in middle-late adulthood as well (Table 1). Interestingly, despite greater pain perception, older patients were more engaged in social life and traveling and more oriented toward personal care and hygiene.

On average, taking all the patients irrespective of gender, the proportion of the physically disabled was about 2.7-fold higher in the group

Table 1 Scoring of complaints in patients with lumbosacral spine pain, stratified into the early and middle-late adulthood age, according to the revised Oswestry Low Back Pain Disability Scale

	Early adulthood ($n = 34$)	Middle adulthood ($n = 46$)	p
Age (year)	31.8 ± 5.2	59.2 ± 10.1	<0.001
Pain	0.89 ± 0.76	2.78 ± 1.18	<0.001
Pain changeability	1.14 ± 1.12	2.56 ± 1.27	<0.001
Personal care	0.77 ± 0.88	1.82 ± 1.47	<0.003
Lifting items	0.94 ± 0.80	2.73 ± 1.16	<0.001
Walking	0.66 ± 0.84	2.00 ± 1.31	<0.001
Sitting	0.94 ± 0.87	2.33 ± 1.09	<0.001
Standing	0.86 ± 0.81	2.69 ± 1.40	<0.001
Sleep problems	0.57 ± 0.61	2.00 ± 1.33	<0.001
Social life	0.91 ± 0.82	1.78 ± 1.55	<0.030
Traveling	0.91 ± 0.82	2.11 ± 1.13	<0.001

Data are means ±SD

Table 2 Proportions of all patients with disability due to lumbosacral spine pain, stratified by age

Age group	$\bar{x}$ ±SD	Min	Q_1	Me	Q_3	Max	p
Early adulthood ($n = 34$)	17 ± 9%	0%	12%	18%	24%	36%	<0.001
Middle-late adulthood ($n = 46$)	46 ± 17%	20%	30%	44%	60%	80%	

Data are $\bar{x}$ ±*SD*, mean ± standard deviation
Min, minimum, *Q1* quartile 1, *Me* median, *Q3* quartile 3, *Max* maximum

Table 3 Proportions of patients with disability due to lumbosacral spine pain, stratified by age and gender

	$\bar{x}$ ±SD	Min	Q_1	Me	Q_3	Max	p
Early adulthood ($n = 34$)							
Women ($n = 15$)	12 ± 11%	0%	5%	4%	21%	30%	0.231
Men ($n = 19$)	15 ± 8%	0%	10%	14%	19%	35%	
Middle-late adulthood ($n = 46$)							
Women ($n = 25$)	41 ± 22%	10%	20%	30%	46%	70%	0.223
Men ($n = 21$)	48 ± 18%	20%	25%	49%	65%	80%	

Data are $\bar{x}$ ±*SD*, mean ± standard deviation
Min minimum, *Q1* quartile 1, *Me* median, *Q3* quartile 3, *Max* maximum

of older patients than that in early adulthood ($p < 0.001$) (Table 2). The older age-dependent predominance of disabilities was further accentuated when the patients were stratified by gender; 48 ± 18% older vs. 15 ± 8% younger men and 41 ± 22% older vs. 12 ± 11% younger women. However, there were no gender-specific differences in either age group of patients (Table 3). That result may somehow seem at variance with other epidemiological studies that point to a higher prevalence of low back pain in women, compared with men (Bento et al. 2019; Jiménez-Trujillo et al. 2019; Wáng et al. 2016). Some previous studies also draw attention to the association between female gender and low back pain. Damage to the intervertebral discs, often reported during menopause, has been pointed out as a factor that predisposes to lumbosacral spine pain complaints (Wáng 2015). However, a direct comparison across various studies is hardly meaningful due to different populations studied and different tools of assessment employed in each. Attention is also paid to muscular weakness and obesity as factors underscoring the occurrence of spinal pain in postmenopausal women, although this may be a coexisting phenomenon rather than a risk factor for pain syndromes (Toda et al. 2000).

Table 4 Proportions of patients with disability due to lumbosacral spine pain, stratified by age and the type of work

	$\bar{x} \pm$SD	Min	Q_1	Me	Q_3	Max	*p*
Early adulthood ($n = 34$)							
Blue-collar	37 ± 19%	9%	20%	30%	50%	72%	0.302
White-collar	30 ± 20%	0%	14%	27%	38%	60%	
Mixed work	33 ± 22%	0%	17%	23%	48%	76%	
Middle-late adulthood ($n = 46$)							
Blue collar	34 ± 20%	7%	20%	31%	56%	75%	0.320
White collar	30 ± 18%	0%	15%	26%	36%	61%	
Mixed work	36 ± 23%	0%	17%	25%	58%	80%	

Data are $\bar{x} \pm SD$, mean ± standard deviation
Min, minimum, *Q1* quartile 1, *Me* median, *Q3* quartile 3, *Max* maximum

The nature of the patients' work, be it blue-collar or white-collar work, failed to exert any appreciable effect on the presence of disability linked to the low back ailment and pain, irrespective of patients' age. The proportion of disabilities in all types of occupation ranged between 30% and 40% (Table 4).

The mean leisure time physical activity, assessed with the MLTPAQ questionnaire, amounted to 5.0 ± 1.4 MET in the early adulthood group and to 4.8 ± 1.7 MET in the middle-late adulthood group of patients; the difference between the two groups was insignificant ($p > 0.05$). Likewise, BMI failed to differ significantly between the two groups of patients, despite a tendency for a somehow higher BMI in the middle-late adulthood group; 23.5 ± 2.7 vs. 25.5 ± 3.2 kg/m^2, respectively.

In contradistinction to the nature of the patients' work, the level of their leisure time physical activity and of BMI associated significantly with the appearance of disability due to the low back pain predicament. The associations were adverse in case of leisure time activity in both early adulthood and middle-late adulthood age groups ($r = -0.536$ and $r = -0.659$, respectively; $p < 0.001$) and positive in case of BMI ($r = 0.686$ and $r = 0.587$, respectively; $p < 0.001$) (Table 5). These findings are, generally, in line with other literature reports. Physical activity has been reported to decrease the risk of self-reported low back pain, particularly in the middle-late-aged persons (Park et al. 2018). Moreover, physical activity is considered to ameliorate the burden of persisting low back pain (Amorim et al. 2019). Many authors point to obesity as a risk factor for low back pain, although some others put in doubt that there is strong evidence for a causal relationship between obesity and back pain (Dario et al. 2015; Leboeuf-Yde et al. 1999). The present findings seem to lend support for the former notion, as we found that BMI associates with the appearance of low back pain complaints. Further, this association comes to light already in the upper part of the BMI norm. We found, however, that the associations of both leisure time physical activity and BMI with the appearance of disabling low back pain were independent of gender and age of the patients investigated.

Table 5 Associations between physical activity, assessed with the Minnesota Leisure Time Physical Activity Questionnaire (MLTPAQ), and body mass index (BMI) with the appearance of disability in patients with lumbosacral spine pain, stratified by age groups

	n	*r*	*p*
Early adulthood ($n = 34$)			
Leisure time physical activity	34	0.536	**<0.001**
BMI	34	0.686	**<0.001**
Middle-late adulthood ($n = 46$)			
Leisure time physical activity	46	0.659	**<0.001**
BMI	46	0.587	**<0.001**

Spearman's rank-order correlation tests

The findings of this study confirm that patients in the middle-late adulthood suffer more frequently from low back pain than do patients in the early adulthood period of life. The ailment is manifest in a greatly enhanced expression of symptoms, particularly in the musculoskeletal,

motion-related realm in the older compared to earlier-aged patients, leading, on average, to about threefold greater appearance of disability in patients in the middle-late adulthood. Despite the obvious symptoms of dysfunction of daily life activities, the older patients showed well-preserved or even enhanced social sphere of functioning. That might have to do with a desire to overcome the physical encumbrance by older adults, which also was reflected in their having a comparable metabolic equivalent of task to that of younger adults.

We further found that the level of disability due to low back pain, assessed by the Oswestry Low Back Pain Disability scale, was basically uninfluenced by other possibly intervening factors investigated, such as gender, type of work, BMI, or leisure time physical activity. We conclude, therefore, that the present findings strongly suggest that older age per se was a major detriment leading to enhanced propensity for the appearance of low back pain. It seems that structural spine damage that naturally and inevitably occurs as a result of normal wear with age is an essential underlier of low back pain complaints, all else unchanged. Low back pain continues to be a scourge that is in the top ten most common health issues in the developing world (Lee et al. 2019; Green et al. 2018). Considering the biopsychosocial burden and the costs of medical services engaged in the management of spinal pain syndromes, further epidemiological insights into the underlying mechanisms and risk factors are desirable in order to undertake effective preventive and curative measures.

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in the study.

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Sex Hormones Response to Physical Hyperoxic and Hyperbaric Stress in Male Scuba Divers: A Pilot Study

Vittore Verratti, Danilo Bondi, Tereza Jandova, Enrico Camporesi, Antonio Paoli, and Gerardo Bosco

Abstract

The use of hyperbaric oxygen plays a significant role in many aspects of medicine. However, there are few studies that analyzed the role of hyperbaric oxygen, in addition to physical exercise, on the endocrine profile. The aim of this study was to compare changes in plasma male sex hormones after hyperbaric physical exercise with different hyperbaric oxygen pre-conditionings. We recruited six healthy, well-trained recreational male divers. Concentrations of prolactin (PRL), follicle-stimulating hormone (FSH), luteotrophic hormone (LH), cortisol, 17-β estradiol (E2), and total testosterone (TT) were measured in venous blood immediately after four different study conditions. Exercise increased PRL and hyperbaric oxygen potentiated this effect. Hyperbaria stimulated the E2 reduction and hyperoxia partially inhibited this reduction. Hyperbaria, but not hyperoxia, stimulated the TT reduction. There were no changes in FSH, LH, and cortisol. The increase in PRL likely reflects a stress response after physical exercise, amplified by hyperbaric oxygen. TT reduction may be interpreted as an acute and transient fertility impairment. Age, blood pressure, and BMI were taken into account as covariates for statistical analyses, and they significantly affected the results, in particular TT. These data open new insight into the role of E2 and PRL in male endocrine adaptive responses.

Keywords

Diving · Exercise · Hyperbaria · Hyperoxia · Scuba divers · Sex hormones · Stress

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1 Introduction

Internal and external stressors challenge the stress system to regulate homeostasis by means of neuroendocrine hormones (Chrousos 2009). Hormonal and neural pathways reciprocally communicate in the paradigm of brain-body interactions. Several stressors, such as adverse childhood experiences or unhealthy lifestyles,

affect physiological pathways in a gender-dependent manner through neuroendocrine, immune, and metabolic mediators (McEwen et al. 2015). Other stressors, such as alterations in oxygen supply and hypo-hyperbaric conditions, are methodologically well established (Mrakic-Sposta et al. 2017; Lund et al. 1999). In particular, humans have mechanisms to counter hypoxia stress (Verratti et al. 2009; Di Giulio et al. 2006), but they lack effective protective systems to counter hyperoxia. The mechanisms of oxygen sensing physiology are an area of still limited understanding (Pokorski et al. 2016). It is known that both high and low oxygen availability may endanger the stability of the redox system (Mrakic-Sposta et al. 2017). Hyperbaric oxygen (HBO) therapy can effectively treat various conditions, such as nonhealing wounds, decompression sickness, or carbon monoxide poisoning, through the enhancement of pro-oxidative status. The effect of hyperbaria on hormonal status is not fully clear.

Physical exercise is considered a stimulant for the endocrine system. Thus, physical activity could be exploited to study hormonal responses in extreme conditions, despite the multifarious typology, intensity, volume, timing, and environmental conditions of exercise (Cano Sokoloff et al. 2016; Hackney and Lane 2015). Scuba diving is a kind of physical activity that is suitable for studies of hormonal responses to hyperbaric conditions since changes in hematologic parameters of healthy scuba divers after diving do not represent any clinical or pathological threat (Perovic et al. 2017). A combination of scuba diving and hyperoxia has already been used as a protocol to test the effect of hyperbaric oxygen on hormonal stress (Weist et al. 2012; Lund et al. 1999). In addition to the adequacy of the model, scuba diving practice is well defined regarding medical aspects, such as decompression illness, but is poorly defined regarding the dynamics of physiological pathways, such as the endocrine system. There are only a few studies that have analyzed the role of oxygen in the endocrine profile and in male fertility. For instance, a reduction in ambient oxygen at high altitude adversely affects spermatogenesis and male fertility (Verratti et al. 2016, 2008). Conversely, hyperbaric oxygen shows a protective and enhancing effect on erectile function after injury to the rat cavernous nerve (Müller et al. 2008).

The androgen/estrogen balance is essential for normal testicular development and for the maintenance of spermatogenesis (Carreau et al. 2007). While the importance of testosterone and gonadotropins in the testicular development and spermatogenesis is an accepted notion, estrogen role in the male physiology remains to be further clarified (van Paridon et al. 2017; Huo et al. 2012; Lavoute et al. 2005; Rogatsky et al. 2005). Evidence suggests an important role of prolactin in male fertility and in the response to acute stressors such as physical exercise. Moreover, it has been found that hyperoxic breathing increases the serum content of prolactin in male athletes (Strüder et al. 1999; Hackney et al. 1989). Therefore, we performed the present study on the premise that different conditions of ambient pressure and oxygen could cause different endocrine alterations. The study addresses the role of hyperbaria, hyperoxia, and light exercise on male sex hormones. The overall aim of the study was to define the modulatory role of physical exercise performed in the hyperbaric condition on the profile of male sex hormones.

2 Methods

This study was conducted as part of the project related to the effects of oxygen prebreathing on scuba divers (Morabito et al. 2011; Bosco et al. 2010). Six healthy volunteers, well-trained recreational male divers, participated in the study. They all were highly experienced diving instructors. Thus, their mental stress due to immersion was at a minimum; they had no osteoarticular or cardiocirculatory pathologies, cancer, or diabetes, nor had they any neurological, psychiatric, respiratory, neuromuscular, and genetic diseases. The subjects' mean age was 38.2 ± 11 years, body mass index (BMI) was 27.1 ± 5.4 kg/m^2, systolic (SBP) and diastolic (BDP) blood pressures were 132 ± 9.8 mmHg and 82.5 ± 6.9 mmHg,

respectively. None of them had diastolic pressure greater than 95 mmHg and systolic pressure greater than 150 mmHg.

The study started with the control condition at rest, during which the subjects breathed ambient air at sea level without exercise and without any changes in the breathing gas mixture. Then, three randomized conditions before underwater exercise followed, as depicted in Fig. 1: I, normoxic exercise, subjects exercised after they breathed ambient air at sea level before diving, 1 standard atmosphere (ATM); II, Oxy-0 m, subjects exercised after they breathed 100% oxygen at sea level before diving; and III, Oxy-6 m, subjects exercised after they breathed 100% oxygen 6 m below the water surface at 1.6 atmosphere absolute (ATA). Exercise was always performed at the bottom of a dive of 30 m depth at 4 ATA in water of 20 ± 5 °C. The subjects breathed with scuba diving gear, consisting of compressed air, during

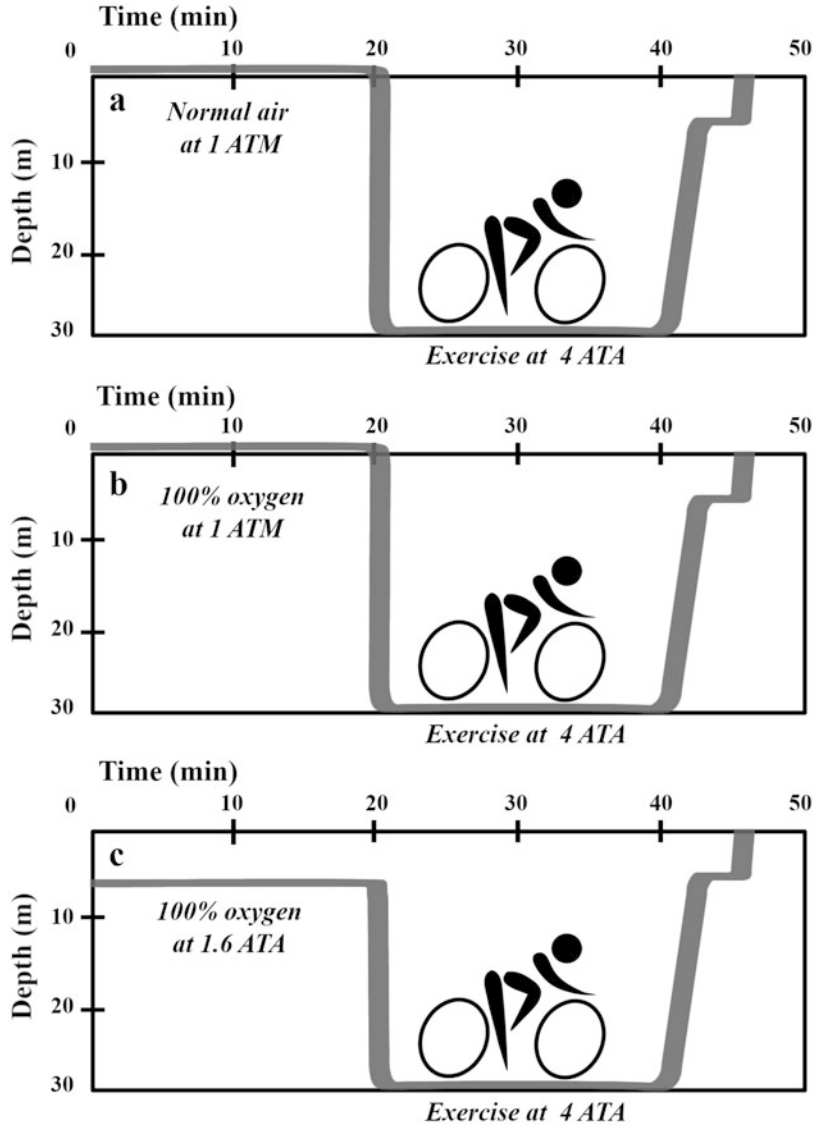

Fig. 1 Study protocol involving conditions with exercise performed 30 m underwater at 4 ATA: (**a**) after breathing ambient air at sea level, (**b**) after breathing 100% oxygen at sea level (Oxy-0 m), and (**c**) after breathing 100% oxygen 6 m below the water surface at 1.6 ATA (Oxy-6 m). *ATM* standard atmosphere, *ATA* atmosphere absolute

20-min long underwater exercise. The depth and duration of immersion were set a priori in order to provide a practical and reliable protocol.

Exercise consisted of the same workload performed on an underwater stationary bicycle (OKEO; Carasco, Italy). The subjects pedaled at a steady rate of 25 rpm to ensure there were no differences in ventilation and gas exchange. The intensity of exercise was set to a moderate level of 3, at the 10-point scale of perceived exertion. The ascent rate from diving was set to 10 m/min, with a 3-min decompression stop at a depth of 5 m. The subjects refrained from taking any medications, diving, and from any other activities for 48 h before each experimental condition. There was a 2-week recovery period between each phase of the protocol. All the experimental conditions were carried out at 9 a.m. in an open sea off the Tremiti Islands in Italy. Free scuba diving was not permitted in order to obtain a standardized exercise protocol. Control values, including blood sampling without exercise or exposure to hyperbaric and hyperoxic conditionings, were established by calculating a mean of three trials performed on three consecutive days prior to the onset of diving protocols.

As soon as the subjects surfaced, and at rest in case of control trials, venous blood samples were drawn from an antecubital vein through a 20-gauge needle. The samples were injected into Vacutainer® tubes (Becton Dickinson; Franklin Lakes, NJ) containing 3.2% trisodium citrate anticoagulant, and it was centrifuged at 3000 rpm at room temperature for 10 min. Then, serum samples were frozen in liquid nitrogen until further use. The samples were essayed for the content of the following hormones in the blood serum: prolactin (PRL – small molecular weight form), 17-β estradiol (E2), follicle-stimulating hormone (FSH), luteotropic hormone (LH), total testosterone (TT), and cortisol, using an ADVIA Centaur® CP Immunoassay System (Siemens Healthcare; Milan, Italy). The system's analytical sensitivity was 0.3 mIU/mL for FSH, 0.1 mIU/mL for LH, 0.3 ng/mL for PRL, 7 pg/mL for E2, and 10 ng/dL for TT.

Data were reported as means ±SD. Data distribution was assessed using the Shapiro-Wilk test. Statistical differences were assessed using repeated measures (RM) ANOVA with the Tukey post hoc correction for multiple comparisons. In addition, assumption checks in the general linear model (GLM) were tested with the Levene test for homogeneity of residual variances and the Kolmogorov-Smirnov test for normality of residuals. Bayesian RM ANOVA, reporting Bayes Factor (BF_{10}), was also used. In both GLM and Bayesian RM ANOVA, we set age, BMI, SBP, and DBP as co-variates. Statistical analyses were carried out using the R-based open-source software Jamovi (https://www.jamovi.org) and Jasp (https://jasp-stats.org).

3 Results

We found a significant increase in PRL from the control level to the diving exposure ($p < 0.001$), with the post hoc significant effects for Control vs. Oxy-0 m ($p < 0.010$) and for Control vs. Oxy-6 m ($p < 0.001$). After controlling for the covariates of age, BMI, SBP, and DBP, the overall effect was reduced, but it remained significant for the diving exposure. The post hoc comparisons reflected this reduction: Control vs. Oxy-0 m – $p = 0.070$ and Control vs. Oxy-6 m – $p = 0.040$ (Table 1 and Fig. 2).

There was a significant decrease in E2 from the control level to the diving exposure ($p < 0.001$), with the post hoc significant effects for Control vs. Exercise ($p < 0.001$) and for Control vs. Oxy-6 m ($p < 0.001$). The Oxy-0 m value of E2 was significantly higher than those of E2 values at Exercise ($p = 0.030$) and Oxy-6 m ($p = 0.010$). After controlling for the covariates, a tendency for the overall effect remained ($p = 0.070$). The post hoc comparisons showed just the borderline significance for Control vs. Exercise ($p = 0.090$) and Control vs. Oxy-6 m ($p = 0.070$) (Table 1 and Fig. 2).

Further, there was a significant decrease in TT from the control level to the diving exposure ($p < 0.001$), with post hoc significant effects for Control vs. Exercise ($p = 0.014$),

Table 1 Statistical evaluation of the relationship of underwater exercise, performed after breathing normoxic normobaric and hyperoxic hyperbaric gas mixtures, to changes in blood content of prolactin (PRL), 17-β estradiol (E2), and total testosterone (TT) in scuba diving volunteers

	PRL			E2			TT		
	p	η^2	BF_{10}	p	η^2	BF_{10}	p	η^2	BF_{10}
RM	<0.001	0.29	42.53	<0.001	0.45	1478.59	<0.001	0.24	30.18
Post hoc									
Control vs. exercise	0.090		1.64	<0.001		2481.14	0.014		3.61
Control vs. Oxy-0 m	<0.010		6.34	0.020		3.52	<0.010		6.94
Control vs. Oxy-6 m	<0.001		5.62	<0.001		47.91	<0.010		12.62
Exercise vs. Oxy-0 m	0.340		8.91	0.030		2.37	0.882		0.54
Exercise vs. Oxy-6 m	0.090		3.21	0.950		0.46	0.583		0.55
Oxy-0 m vs. Oxy-6 m	0.850		0.49	0.01		2.20	0.944		0.50
RM with co-variates									
RM	0.040	0.29	42.67	0.070	0.45	1492.59	0.280	0.24	30.15
RM∗age	0.620	0.04	58.18	0.670	0.07	1081.98	0.370	0.25	23.32
RM∗BMI	0.520	0.05	40.75	0.900	0.03	1079.47	0.610	0.13	25.34
RM∗SBP	0.820	0.02	31.02	0.930	0.02	1524.98	0.530	0.16	21.30
RM∗DBP	0.350	0.08	208.47	0.520	0.10	1100.74	0.280	0.33	35.43
Post hoc									
Control vs. exercise	0.260		1.64	0.090		2481.14	0.480		3.61
Control vs. Oxy-0 m	0.070		6.34	0.410		3.52	0.360		6.94
Control vs. Oxy-6 m	0.040		5.62	0.070		47.91	0.280		12.62
Exercise vs. Oxy-0 m	0.500		8.91	0.460		2.37	0.970		0.54
Exercise vs. Oxy-6 m	0.260		3.21	0.990		0.46	0.940		0.55
Oxy-0 m vs. Oxy-6 m	0.890		0.49	0.360		2.20	0.990		0.50

Control, breathing ambient air at rest at sea level. Diving conditions: I, exercise underwater after breathing ambient air at sea level before diving; II, Oxy-0 m, exercise underwater after breathing 100% oxygen at sea level before diving; and III, Oxy-6 m, exercise underwater after breathing 100% oxygen 6 m below the water surface at 1.6 atmosphere absolute (ATA). Exercise was in all cases performed at the bottom of a dive of 30 m below water surface. *RM*, repeated measures. Covariates: *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure. p statistical significance, η^2 effect size; and BF_{10} Bayesian factor

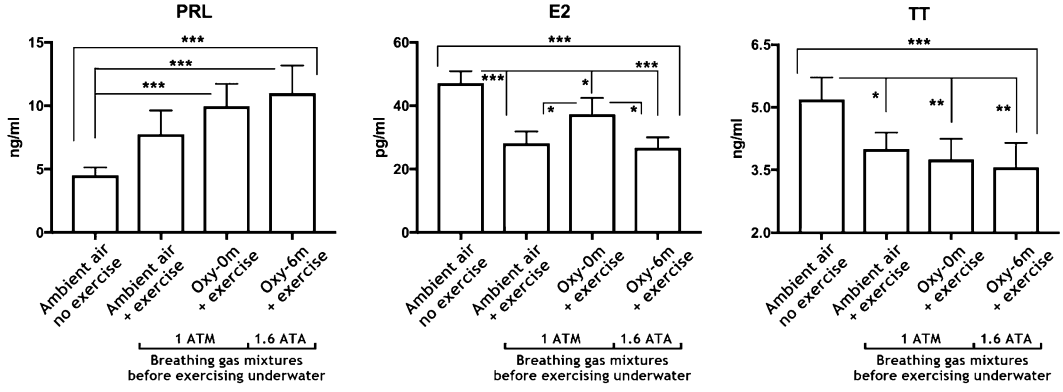

Fig. 2 Changes in blood content of prolactin (PRL), 17-β estradiol (E2), and total testosterone (TT) after underwater exercise performed after breathing ambient air at sea level, 100% oxygen at sea level (Oxy-0 m), and after breathing 100% oxygen 6 m below the water surface at 1.6 atmosphere absolute (ATA) in scuba diving volunteers (Oxy-6 m). *ATM*, standard atmosphere. Data are means ±SE; $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$

Control vs. Oxy-0 m ($p < 0.010$), and Control vs. Oxy-6 m ($p < 0.010$). However, after controlling for the covariates, we failed to show any statistical significance or tendencies (Table 1 and Fig. 2). There were no other significant differences substantiated for FSH, LH, or cortisol contents, but a tendency for the increment in FSH from the control level to diving exposure was noticed ($p = 0.080$), which disappeared after controlling for the co-variates (Table 2).

4 Discussion

The present study examined the physiological effects on the homeostasis of the endocrine system of three different stressors, consisting of a combination of physical exercise, hyperoxia, and hyperbaria. The findings were that the stressors significantly increased the content of PRL and decreased those of E2 and TT in the venous blood. In detail, hyperbaric physical pre-conditioning increased the content of PRL, and hyperoxia pre-conditioning potentiated the effect in a pressure-dependent manner (Fig. 2). Further, these effects were present, albeit slightly attenuated but still with a coherent trend, even after controlling for age, BMI, SBP, and DBP, with slight attenuation (Table 1).

It is known that PRL content increases as a result of physical activity, with a simultaneous reduction in testosterone, FSH, and LH (Mićić et al. 1985). In the hypothalamo-pituitary axis, PRL is the most dynamic hormone. Apart from the hormone's key role in pregnancy and lactation, it takes part in a range of other bodily functions (Grattan 2015). Anegg et al. (2002) have demonstrated that the diving-related changes in PRL are due also to emotional stress. A half-life of PRL is lower than that for cortisol. PRL may thus be considered a fast-response stress marker.

PRL synthesis and release are regulated by the hypothalamic factors in a dichotomous manner: thyrotropin-releasing hormone (TRH), serotonin, estrogens, and endorphins facilitate the release of PRL, whereas gamma-aminobutyric acid (GABA) inhibits its secretion. Kennett and McKee (2012) have proposed a dualistic model of stimulation of PRL secretion, with oxytocin on the positive side and dopamine (DA) on the negative one. DA has an established role in the control of PRL secretion with a short-loop feedback. Hyperoxia reduces DA level (Adachi et al. 2001), and this reduction seems an acute rather than chronic, due to continual exposure, effect (Lavoute et al. 2005). These previous studies have demonstrated that oxygen influences DA metabolism and secretion in the extracellular striatal space. Presumably, normobaric and hyperbaric hyperoxia leads to a significant inhibition of DA release in the striatum, which controls PRL secretion (Spencer et al. 2011). Dopaminergic drugs that alter PRL content also produce antiestrogenic effects as demonstrated in the mouse uterus (Gunin et al. 2002). Hyperbaric hyperoxia may thus be considered a key factor in the regulation of dopaminergic inhibition of PRL secretion.

The present findings demonstrate that in all the diving protocols, E2 blood content was lower than that in the control condition. In the hyperoxic diving protocols, interestingly, the E2 reduction was attenuated (Fig. 2); the attenuation was smaller after controlling for age, BMI, SBP, and DBP (Table 2). The hyperbaric condition also was related to a lower level of TT. In this case hyperoxia failed to influence this trend, and the effect was strongly attenuated after controlling for age, BMI, SBP, and DBP. The influence of PRL on the E2 and TT reduction during hyperbaric oxygenation seems a viable plausibility, taking into account a strong regulatory action of PRL on other hormones above outlined.

The general role of testosterone linked to physical fitness is well established (DeFina et al. 2018), although data on the response of testosterone to exercise are contentious, which may have to do with unstandardized experimental protocols, therapies of the animal and human models involved. There are data demonstrating an increase in blood testosterone after hyperbaric oxygen therapy in humans (Passavanti et al. 2010), but an acute decrease in testosterone has been observed after hyperbaric conditioning and diving in rats (Röckert et al. 1978). Further, lack of a change in testosterone content has also been

Table 2 Statistical evaluation of the relationship of underwater exercise, performed after breathing normoxic normobaric and hyperoxic hyperbaric gas mixtures, to changes in blood content of follicle-stimulating hormone (FSH), luteotropic hormone (LH), and cortisol in scuba diving volunteers

	FSH			LH			Cortisol		
	p	η^2	BF_{10}	p	η^2	BF_{10}	p	η^2	BF_{10}
RM	0.08	0.02	1.21	0.66	0.04	0.30	0.38	0.14	0.37
Post hoc									
Control vs. exercise	0.24		0.99	0.84		0.52	0.90		0.60
Control vs. Oxy-0 m	0.07		0.97	0.81		0.52	0.43		0.43
Control vs. Oxy-6 m	0.24		1.33	0.99		0.38	0.99		0.37
Exercise vs. Oxy-0 m	0.88		0.64	0.99		0.37	0.82		0.55
Exercise vs. Oxy-6 m	0.99		0.37	0.80		0.44	0.89		0.63
Oxy-0 m vs. Oxy-6 m	0.88		0.52	0.77		0.58	0.42		0.59
RM with covariates									
RM	0.57	0.01	1.20	0.77	0.04	0.30	0.92	0.04	0.37
RM∗age	0.99	0.01	1.24	0.92	0.03	0.35	0.90	0.08	0.33
RM∗BMI	0.21	0.06	1.23	0.52	0.12	0.19	0.38	0.42	0.47
RM∗SBP	0.55	0.02	1.12	0.72	0.07	0.18	0.96	0.04	0.26
RM∗DBP	0.32	0.04	2.12	0.98	0.01	0.90	0.85	0.10	0.25
Post hoc									
Control vs. exercise	0.74		0.99	0.89		0.52	0.93		0.60
Control vs. Oxy-0 m	0.54		0.98	0.88		0.52	0.99		0.43
Control vs. Oxy-6 m	0.74		1.33	0.99		0.38	0.99		0.37
Exercise vs. Oxy-0 m	0.98		0.64	0.99		0.37	0.99		0.55
Exercise vs. Oxy-6 m	0.99		0.37	0.86		0.44	0.93		0.63
Oxy-0 m vs. Oxy-6 m	0.98		0.52	0.84		0.58	0.99		0.59

Control, breathing ambient air at rest at sea level. Diving conditions: I, exercise underwater after breathing ambient air at sea level before diving; II, Oxy-0 m, exercise underwater after breathing 100% oxygen at sea level before diving; and III, Oxy-6 m, exercise underwater after breathing 100% oxygen 6 m below the water surface at 1.6 atmosphere absolute (ATA) in scuba diving volunteers. Exercise was in all cases performed at the bottom of a dive of 30 m below water surface. *RM*, repeated measures. Covariates: *BMI* body mass index, *SBP* systolic blood pressure, *DBP* diastolic blood pressure. p statistical significance, η^2 effect size; and BF_{10} Bayesian factor

reported after hyperbaric oxygen therapy in rats (Nakada et al. 1986) or after a brief free diving (Jóźków et al. 2012). A reduction in testosterone is a clear marker of male fertility impairment. Thus, acute testosterone reduction we found in the present study may be due to the effect of hyperbaria on testicular perfusion, rather than that of exercise or hyperoxia.

In the present study, FSH and LH did not change in the diving protocols. These results are in line with those of Jóźków et al. (2012) who have investigated changes in the content of gonadotropins after brief free diving. The content of cortisol did not change either, which suggests that the level of exercise was moderate, which was also confirmed by the subjects' perception of exertion, nor was there any emotional stress involved, otherwise present during competitions (van Paridon et al. 2017). Concerning the changes in cortisol during exercise and diving, the literature is discordant. For instance, Weist et al. (2012) have reported no change in the content of cortisol after hyperoxic or hyperbaric conditions, while Zarezadeh and Azarbayjani (2014) have reported an increase in it as a result of diving. Therefore, reactivity of cortisol to scuba diving and hyperbaric oxygen remains to be further explore, taking into account also the role of psycho-emotional state and metabolic distress (Joseph and Golden 2017).

This study had some limitations. The number of participants was rather small. However, considering the study protocol, including medical assessments, equipment, resources, and diving

practice, a wider participation in this kind of study was hardly achievable. Further, it could be meaningful to assess recent sexual activity of participants. In addition, it would be reasonable to obtain also pre-diving values for each protocol in order to assess hormonal reactivity. Yet, considering the possible interference of pre-diving blood drawing on the subsequent immersion, we chose to establish only one control value of the hormonal indices measured, taking the average of three basal measurements on three consecutive days instead.

5 Conclusions

Scuba diving represents an interesting model to investigate the endocrine adaptive responses to hyperbaric oxygen and physical conditioning from both physiological and therapeutic point of view. In this study we evaluated the serum profile of sex hormones involved specifically with male fertility, such as gonadotropins, testosterone, and estrogens (Verratti et al. 2016; Carreau et al. 2007) during physical exercise performed by scuba divers at a depth of 30 m after breathing normoxic and hyperoxic gas mixtures in normo- and hyperbaric conditions. The major findings consisted of the increment in the level of prolactin in response to exercise, which was potentiated by hyperoxic preconditioning, and a reduction in 17-β estradiol after light hyperbaric exercise. These findings provide insights into the role of both hormones in the male adaptive endocrine responses. Prolactin, in particular, may be seen as a mediator of the effects on testosterone metabolism of physical exercise, hyperbaria, and hyperoxia. On the other hand, significance of a reduction in 17-β estradiol, in response to hyperbaria, should be subject to further studies. Considering that acclimatization normalizes the high altitude-induced alterations in the adrenal and gonadal endocrine axes (von Wolff et al. 2018), further exploration is required concerning the effects of acute and chronic differences in endocrine dynamics when hyperoxia and hyperbaria are combined with physical exercise. Finally, hormonal reactivity related to scuba diving should also be clarified in relation to the potential impairment of cognitive functions after immersions (Pourhashemi et al. 2016).

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The experimental procedures were approved by the Ethics Committee for Human Research of the University of Chieti in Italy.

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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Cigarette Smoke-Induced Oxidative Stress and Autophagy in Human Alveolar Epithelial Cell Line (A549 Cells)

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Abstract

Chronic exposure to cigarette smoke (CS) causes structural and functional changes in the respiratory tract. It is a major risk factor for cardiovascular and systemic pulmonary diseases. The aim of this study was to investigate the effect of acute CS exposure (2 h) on oxidative stress, heat shock protein 70 (HSP70) expression, autophagy (LC3 expression), and oxidative stress (DCF fluorescence) in human alveolar epithelial cell line A549. Cell culture medium was conditioned with CS using commercial cigarettes, and A549 cells were grown in modified media for 2 h. In some experiments, A549 cells were pretreated with 100 μM of L-buthionine-sulfoximine (BSO) for 24 h to induce glutathione (GSH) depletion. In the cells grown in CS-conditioned medium, GSH was depleted by more than 30%, and reactive oxygen species were increased. Moreover, there was a considerable overexpression of HSP70 and a substantial accumulation of LC3. Similar changes were found when the cells were pretreated with BSO. We conclude that the short-term exposure of epithelial cells to CS increases oxidative stress that entails enhanced autophagy activity.

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Keywords

A549 cell line · Autophagy · Cigarette smoke · Heat shock protein · Oxidative stress

1 Introduction

Cigarette smoke (CS) is a major risk factor for cardiovascular and systemic respiratory tract diseases and is considered to be a leading cause of death in the world (Van Eeden et al. 2012). CS contains more than 5000 chemicals, which have toxic, mutagenic, and carcinogenic effects (Talhout et al. 2011). Chronic CS exposure causes structural and functional changes in the respiratory tract, which are involved in the development of chronic obstructive pulmonary disease (COPD), epithelial cell tumors, and cardiovascular diseases but also increase the incidence of asthma and respiratory infections (Barnes 2014). The mechanisms of CS toxicity are complex, but protein damage and DNA fragmentation are the hallmarks of CS-induced changes (Carnevali et al. 2003). CS can also promote inflammatory response through the activation of many signaling pathways involving mitogen-activated protein kinases (MAPKs) or nuclear factor kappa-B (NF-κB) (Rom et al. 2013). On the other hand, CS disturbs the pro-oxidant–anti-oxidant balance leading to cellular damage in the respiratory tract. It contains and generates various reactive oxygen

species (ROS) and reactive nitrogen species (RNS), such as superoxide radical, hydrogen peroxide, hydroxyl radical, and peroxynitrite (Faux et al. 2009). CS-derived ROS cause cell growth arrest, cell detachment, and cell lysis and affect epithelial permeability (Aoshiba and Nagai 2003).

Autophagy is a cytoprotective mechanism which can be triggered by different stimuli, including oxidative stress, protein aggregates, and toxic molecules to mitigate stress. The role of autophagy in cell viability is complex, but the process can be activated in response to stress, and it has been identified as a critical modulator of tissue damage (Saha et al. 2018). Oxidative stress is a major driving force for autophagy. Therefore, autophagy could protect against the harmful effects of oxidative damage. It has been demonstrated that dysfunction of autophagy is also involved in numerous pathophysiological processes, such as cancer or cardiovascular and pulmonary diseases (Malaviya et al. 2014).

We have previously shown that 24-hour exposure of human alveolar epithelial cells (A549 cells) to CS activates internal and external inflammatory responses and cytokine secretion. A key cytokine responsible for pro-inflammatory signaling is interleukin (IL)-1β (Holownia et al. 2016). The purpose of the present study was to investigate the effect of acute CS exposure on oxidative stress, heat shock protein 70 (HSP70) expression, and autophagy in A549 cells.

2 Methods

2.1 Cell Culture

A549 (ATCC® CCL185™) cells were grown in ATCC-formulated F12K medium supplemented with 10% fetal bovine serum. The cells were maintained in 37 °C in an incubator in a humidified atmosphere containing 5% CO_2. For the experiments, cells were plated out onto six-well plates and were grown in control or smoke-conditioned media for 2 h. In some experiments, cells were pretreated with 100 μM of L-buthionine-sulfoximine (BSO) for 24 h to deplete glutathione (GSH).

2.2 Preparation of Cigarette Smoke (CS)-Conditioned Media and Cell Treatment

CS-conditioned medium (CSM) was prepared using full-strength Red Marlboro cigarettes (Phillip Morris, Cracow, Poland) containing 8 mg of tar, 0.6 mg of nicotine, and 9 mg of carbon monoxide per cigarette. Cigarette filters were removed, and smoke was passed through the culture media (two cigarettes per 50 ml of medium), using a low-pressure vacuum pump. The CS-conditioned medium was then filtered, using 0.22 μm filters, and was applied immediately to cell culture. The nicotine content in the CS-conditioned medium was about 0.1 mg/ml (GC-MS analysis). A549 cells were grown in the media for 2 h.

2.3 Autophagy Determination

An autophagy marker, LC3 protein, which is recruited to the double membrane of autophagosome, was used to quantify autophagy. LC3 probes were specific monoclonal LC3A/B rabbit antibodies conjugated to Alexa Fluor 647 (Cell SignalingTechnology; Danvers, MA). To quantify LC3 protein, A549 cells were immunostained for 1 h on ice with specific antibodies, washed twice with incubation buffer, and evaluated using flow cytometry (Epics XL; Coulter Electronics, High Wycombe, UK).

2.4 Determination of Heat Shock Protein (HSP70)

A549 cells were incubated with mouse anti-HSP70 antibody (1:100) (Abcam; Cambridge Biomedical Campus, Cambridge, UK) for 1 h at 37 °C, followed by incubation with Alexa Fluor 488-conjugated anti-mouse antibody. HSP70 protein content was determined using the Epics XL cytometer.

2.5 Cellular Reactive Oxygen Species (ROS)

Reactive oxygen intermediates were quantified using dichlorodihydrofluorescein diacetate (H_2DCFDA) (Sigma-Aldrich; St. Louis, MO). A549 cells were loaded with 5 μM H_2DCFDA for 30 min, washed, resuspended in phosphate-buffered saline, and assayed by flow cytometry. Green dichlorofluorescein (DCF) fluorescence was captured on F11 channel of the Epics XL flow cytometer, and it was registered as histograms of fluorescence distribution.

2.6 Glutathione Assay

The GSH/GSSG content was assessed using a glutathione assay kit (Cayman Chemical Company; Ann Arbor, MI), based on the enzymatic recycling method with glutathione reductase. A549 cells were harvested, and the whole cell extract was prepared according to the manufacturer's instructions. Contents of GSH and GSSG were determined photometrically at 405 nm using a microplate reader, and the GSH/GSSG levels were calculated.

2.7 Statistical Analysis

Data were expressed as means ±SD of four to six assays. Statistical differences were evaluated using one-way or two-way analysis of variance (ANOVA) followed by a post hoc Bonferroni correction for selected pairs of data. A p-value <0.05 defined statistically significant differences. The evaluation was performed with a commercial Statistica 6.0 package (Statsoft; Cracow, Poland).

3 Results

Flow cytometry of H_2DCFDA-labeled A549 cells showed that 2-hour exposure to CS caused a twofold increase in cellular oxidative stress as compared with control cells ($p < 0.01$). A similar effect was observed in the cells pretreated with BSO. GSH constitutes the first-line defense against a large number of reactive species found in CS. We further found that exposure of A549 cells to CS decreased the GSH content by more than 30%, compared to that in the control cells. In the BSO-treated cells, GSH was reduced level to a similar extent (Fig. 1a, b). We also found that exposure of A549 cells to CS increased expression of LC3 protein, which is the essential protein for autophagosome formation, compared to that in the control cells (Fig. 1c) ($p < 0.01$). Likewise, pretreatment of A549 cells with BSO increased both the level of oxidative stress and the accumulation of LC3 protein, the latter by more than twofold (Table 1).

We subsequently evaluated the influence of CS exposure on HSP70 expression in A549 cells. CS caused a 15% increase in HSP70 ($p < 0.05$) (Fig. 1d), while pretreatment of the cells with BSO produced even a more pronounced effect and increased the HSP70 level by more than 40% ($p < 0.01$) (Table 1).

4 Discussion

The epithelial lining of airways forms a barrier against environmental air pollutions, notably, against CS. Destruction and increased permeability of the epithelial barrier expose subepithelial layers to toxic agents (Aghapour et al. 2018). The epithelial cells play an important role in inflammatory responses by releasing chemokines and cytokines, which are involved in the recruitment and subsequent activation of inflammatory cells. These agents are also engaged in the production of ROS as a result of oxidant/anti-oxidant imbalance, which contributes to chronic pulmonary inflammation and lung tissue damage. Numerous studies have indicated that epithelial cell damage play a crucial role in the development of COPD (Gao et al. 2015; Thorley and Tetley 2007).

Most studies investigating the role of CS in the pathophysiology of COPD have been carried out in chronic smokers. However, acute cigarette smoke exposure can give more specific information about the pathophysiological mechanisms of

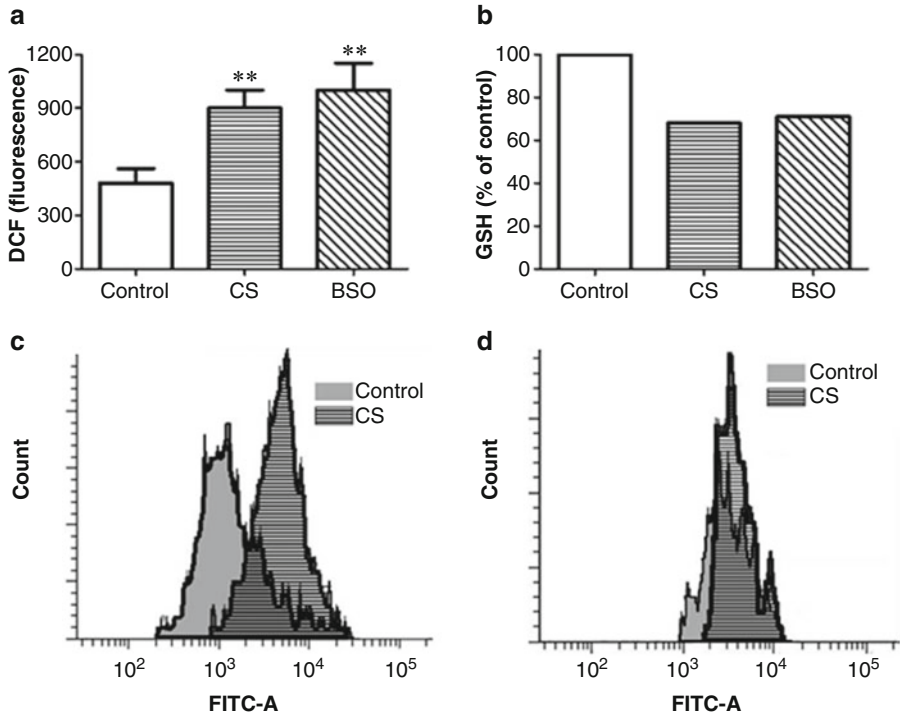

Fig. 1 Effects on oxidative stress of acute (2 h) cigarette smoke (CS) exposure and L-buthionine-sulfoximine (BSO) treatment of A549 line of human alveolar epithelial cells: (**a**) dichlorofluorescein (DCF) assay; (**b**) glutathione (GSH); (**c**) autophagy (LC3 protein expression; and (**d**) heat shock protein 70 expression (HSP70); *FITC-A* fluorescein isothiocyanate mean fluorescence intensity within the A549 cells; $^{**}p < 0.01$ vs. the corresponding control cells

Table 1 The effects of acute (2 h) cigarette smoke (CS) exposure and L-buthionine-sulfoximine (BSO) treatment on oxidative stress – a green dichlorofluorescein (DCF) assay, autophagy (LC3 protein expression), and heat shock protein 70 (HSP 70) expression in human alveolar epithelial cells (A549) evaluated with flow cytometry

	Control	CS	BSO
DCF (mean fluorescence)	480 ± 84	904 ± 107**	1,001 ± 147**
LC3 expression (mean fluorescence)	150 ± 17	246 ± 24**	348 ± 28**
HSP 70 expression (mean fluorescence)	148 ± 8	171 ± 6*	211 ± 14**
GSH (% of control)	100	68	71

GSH glutathione
$^{*}p < 0.05$; $^{**}p < 0.01$ for comparisons with the corresponding control cells

smoking-induced lung disease (Van der Vaart et al. 2004). Thus, the goal of the present study was to investigate a direct impact of acute CS exposure on the redox balance, on HSP70 expression, and on autophagy. In some experiments, we also used BSO to produce GSH depletion in order to assess the role of redox imbalance in CS toxicity.

A549 cells is a cell line, derived from human pulmonary adenocarcinoma, commonly used to investigate the effects of CS or air pollution on the conducting airways (Hiemstra et al. 2018). It has been demonstrated that the impact of CS on A549 cells depends on the smoke concentration and the time of exposure. Hoshino et al. (2001) have demonstrated that CS extract at lower

concentrations induces apoptosis of A549 cells, while at higher concentrations it produces cells' necrosis. We have previously shown that A549 cells grown for 24 h in a CS-conditioned culture medium die due to chemical and oxidative stress (Holownia et al. 2015). We have also shown that 24-h exposure of A549 cells to CS activates internal and external inflammatory responses and cytokine secretion (Holownia et al. 2016). In the current study, we investigated the acute effects of a 2-hour exposure to CS on oxidative stress, heat shock protein 70 (HSP70) expression, and autophagy.

Autophagy is a protective process that captures, degrades, and recycles protein aggregates and intracellular components to maintain metabolic homeostasis. Autophagy impairment has been linked to a wide variety of diseases. It has also been recognized as having a complex impact on the initiation, progression, and treatment of cancer (Ryter and Choi 2015). Autophagy modulates inflammatory and immune processes, and it underlies the early detrimental reaction to CS, all of which is at play in the pathogenesis of COPD (Racanelli et al. 2018). A substantial increase in the autophagy marker proteins, LC3-II and ATG, has been demonstrated in COPD; the increase is notably evident in disease progression (Chen et al. 2008).

In the present study, we showed that short CS exposure increased autophagy in A549 cells. This effect could be associated with CS-induced oxidative stress, which is known to induce both apoptosis and autophagy (Filomeni et al. 2015). Autophagy can act as a protective cellular response, but it is also implicated in the regulation of cell death. Autophagic proteins play a functional role in the degradation of epithelial cell in response to CS through interactions with apoptotic proteins, in particular, by activating the extrinsic apoptotic pathway (Chen et al. 2010). CS-induced oxidative stress plays a role in alveolar wall destruction, leading to airway enlargement (Kode et al. 2006). Moreover, it releases pro-inflammatory cytokines whose content increases in the lungs of smokers and COPD patients (Rahman et al. 2006). GSH is a major anti-oxidant in airway epithelial cells, where it inactivates ROS and detoxicates lipid peroxides and other toxic metabolites. A primary function of GSH is to maintain redox homeostasis, which minimizes oxidation of critical cysteine residues in cellular proteins (Gould et al. 2015). In this study we showed that acute exposure of A549 cells to CS, also those pretreated with BSO, increased ROS production and depleted GSH. These findings are consistent with previous reports. Zhang et al. (2017) have found that CS induces lipid peroxidation and redox imbalance of GSH and it upregulates extracellular superoxide dismutase and oxidative damage of DNA in A549 and BEAS-2B cells. Moreover, CS exposure increases malonyldialdehyde and 4-hydroxy-2-nonenal content; the latter increases in A549 cells in proportion to CS content. Likewise, Kode et al. (2006) have found that CS extract decreases GSH content in a dose-dependent manner in A549 cell line. In contradistinction, in primary human small airway epithelial cells, there is a dose-dependent increase of GSH in response to CS during a 24-h period, which may represent a rebound compensatory effect due to upregulation of glutamate cysteine ligase.

In this study, acute CS exposure increased the expression of HSP70 proteins. These proteins are important chaperones, localized intracellularly or extracellularly, which maintain the cell viability. The intracellular HSP70 exerts an anti-inflammatory effect, while the extracellular one is considered a molecule having a pro-inflammatory immunomodulatory effect (Edkins et al. 2018). HSP70 is implicated in the pathogenesis of COPD, but its role has not been fully elucidated. Previous reports evaluating HSP70 expression in cells exposed to CS have provided contentious findings. These proteins depend on the smoking status, and they decrease in leukocytes from COPD patients (Rumora et al. 2008). However, most of the in vitro studies have shown that CS is a potent inducer of HSP70 protein synthesis in different cell types. Somborac–Bačura et al. (2018) have found an increase in HSP70 expression after the incubation of A549 cells with CS for 6 h; the increase is sustained for up to 48 h. Likewise, Li et al. (2007) have found that HSP70 secretion is induced by CS in a dose- and time-dependent manner in the primary human lung fibroblasts.

HSP70 proteins have a capacity to stabilize lysosomal membranes and to influence autophagy, which is conducive to cancer cell survival (Leu et al. 2009). Therefore, we presume that increased expression of HSP70 in the present study may represent a protective mechanism against CS-induced injury of alveolar epithelial cells. Hulina–Tomašković et al. (2018) have shown that HSP70 proteins modulate the inflammatory response in NCI-H292 cells exposed to CS extract by acting on the interleukin cytokines and the Toll-like receptors (TLR2 and TLR4) and Hsp70 gene expression. These effects take place due to the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB) signaling pathways.

In conclusion, this study supports the view that autophagy is involved in the stress response to cigarette smoke in the airway epithelium. Autophagy plays a protective role at the crossroads between oxidative stress and cell death. Our findings suggest that oxidative stress and HSP70 proteins might be implicated in the development of pulmonary diseases affected by cigarette smoke, such as COPD. We submit that modulating the process of cellular autophagy might become a therapeutic target for the prevention of cigarette smoke-induced pulmonary toxicity.

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

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants or animals performed by any of the authors.

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Relative Cerebral Blood Transit Time Decline and Neurological Improvement in Patients After Internal Carotid Artery Stenting

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Abstract

In this study we hypothesized that the alleviation of neurological symptoms long after internal carotid artery (ICA) stenting may be related to sustained improvement of cerebral perfusion. Thirty-four subjects (F/M; 15/19)

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with >70% stenosis of a single internal carotid artery and neurological symptoms, who underwent a carotid artery stenting procedure, were studied. Brain computed tomography perfusion (CTP) imaging was performed before and 3 years after ICA stenting. The following relative variables were compared: cerebral blood flow (rCBF), cerebral blood volume (rCBV), mean transit time (rMTT), time to peak (rTTP), and permeability surface area product (rPS). A survey also was conducted to compare the patients' clinical symptoms. Overall, we found that a trend toward rMTT decline was the only persisting change after ICA stenting. We then stratified the patients into the subgroups of <2%, 2–5%, and > 5% rMTT decline and found that those with a rMTT decline >2% reported a prominent reduction in subjective clinical symptoms such as headache, dizziness, tinnitus, blurred vision, transient blindness, a sense of gravity of the head, and pain in the eyeballs. We conclude that a shortened mean rMTT, likely reflecting improved cerebral microcirculation, underlies the improvement of neurological symptoms in patients with ICA stenosis.

Keywords

Blood transit time · Cerebral blood flow · Computed tomography perfusion · Internal carotid artery · Neurological symptoms

1 Introduction

Internal carotid artery (ICA) stenosis-related brain hypoperfusion is associated with neurological, psychiatric, and psychological deficits, manifesting as headache, dizziness, tinnitus, blurred vision, transient blindness, a sense of gravity in the head, and pain in the eyeballs. These symptoms associate with cognitive and mood decline. Neuropsychological deficits may not be as apparent as the neurological ones, but they add to the downgrading of quality of life. Numerous studies have demonstrated that ICA stenosis, without transient ischemic episodes or strokes, is a detriment to executive functions and memory (Wang et al. 2016; Romero et al. 2009; Sztriha et al. 2009; Popovic et al. 2011; Mathiesen et al. 2004). It has been demonstrated that cognitive function may improve in symptomatic patients after ICA recanalization, with both endarterectomy and carotid stenting (Yamashita et al. 2012; Chida et al. 2010). Interestingly, Grunwald et al. (2006) have shown cognitive improvements in word fluency and delayed recall in neuropsychological tests conducted 24 h after carotid artery stenting also in asymptomatic patients. Results, akin to those outlined above, have been reported by Wang et al. (2017) and Picchetto et al. (2013) in asymptomatic patients.

ICA stenosis is considered an independent risk factor for stroke (Goessens et al. 2007). It also contributes to the pathophysiology of depression (Rao et al. 2001; Mathiesen et al. 2004; Bakker et al. 2000), and it is a risk factor for suicides in stroke patients (Lovett and Rothwell 2002). According to Huang et al. (2012), ICA stenting relieves post-stroke depression in high-grade ICA stenosis patients over the time of 1 month. A therapeutic effect of stenting is superior to that exerted by selective serotonin reuptake inhibitors in these patients. In contrast, however, Picchetto et al. (2013) have observed no difference between pre- and post-scoring in the neuropsychological evaluation of depression and anxiety carried out in asymptomatic ICA stenosis patients subjected to stenting.

Cerebral blood flow (CBF), which may be assessed during computed tomography perfusion (CTP) examination, improves after ICA stenting (Szarmach et al. 2017; Frydrychowski et al. 2013; Trojanowska et al. 2006; Niesen et al. 2004). However, two other CTP-derived variables, namely, blood-brain barrier (BBB) permeability and mean transit time (MTT), are less recognized in the research and diagnosis of ICA stenosis. The BBB functioning is impaired, and the diffusion of fluid, blood, or contrast molecules into the extravascular space is augmented in the pathologic states such as neoplastic or inflammatory diseases, ischemia, and neurodegenerative disorders (Topakian et al. 2010; Jain et al. 2008). We have demonstrated that elevated BBB permeability can be reversed in

subjects with ICA stenosis after stenting (Szarmach et al. 2017). A disease-enhanced BBB permeability can be considered a radiological marker of neuroinflammation. A decline in BBB permeability with improvement of cerebral blood circulation, and thus increased delivery of oxygen to the brain tissue, suggests that the overall brain ischemic and inflammatory status improves.

Prolongation of MTT is increasingly linked to a cerebral small vessel disease (Arba et al. 2017; Cao et al. 2016). Such diseases, in turn, are associated with the accumulation of immune cells and erythrocytes in the brain microvessel lumina (Kaiser et al. 2014; Mencl et al. 2013; Schreiber et al. 2013; Rouhl et al. 2012; Schreiber et al. 2012). The MTT is prolonged in subjects with ICA stenosis (Szarmach et al. 2017). Importantly, acute MTT decline, 4–6 weeks after ICA stenting, sends a positive signal for the long-term MTT downward response (Winklewski et al. 2019). Thus, MTT decline after stenting strongly suggests the possibility of functional improvement in brain microcirculation.

Several authors have suggested that relative MTT (rMTT), which is the symptomatic hemisphere-to-asymptomatic hemisphere MTT ratio, may represent an early and sensitive parameter for the detection of perfusion changes (Duan et al. 2012; Merckel et al. 2012; Wilkinson et al. 2003). Consequently, we hypothesized that the long-term decline in rMTT after ICA stenting may be interrelated with the alleviation of neurological symptoms reported by patients.

2 Methods

2.1 Patient Population

There were 34 patients (F/M; 15/19) of the mean age of 69.7 ± 7.6 years, suffering from ICA stenosis, investigated during 2010–2014 (Table 1). The patients were qualified for a carotid artery stenting procedure in the Department of Cardiac and Vascular Surgery of the Medical University of Gdansk in Poland.

A general neurological and internal examination of all the patients was performed before the stenting procedure. Patients with stenosis of more than 70% within a single internal carotid artery and with concomitant neurological symptoms (i.e., headache, dizziness, tinnitus, blurred vision, transient blindness, a sense of gravity of the head, and pain in the eyeballs) were included in the study. In all patients, duration of carotid artery stenosis was longer than 5 years. The degree of artery stenosis, according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET 1991), was evaluated using Doppler ultrasonography and confirmed immediately prior to stent implantation by digital subtraction angiography (DSA). Only were patients with a complete circle of Willis and normal vertebral arteries included in the evaluation.

Table 1 Risk factors and concomitant diseases in the population studied

Risk factor/disease	No. of cases
Smoker	15
Hypertension	34
Diabetes mellitus type 2	11
Transient ischemic attack	10
Stroke	5
Coronary artery disease	24
Lipid disorder	11
Myocardial infarction	14

Patients with bilateral hemodynamically significant (i.e., >70%) stenosis of cranial arteries, patients with complete occlusion of one vessel, or those who had experienced extensive ischemic brain stroke were excluded from the study. Consequently, in the patients included in the study, stenosis on the contralateral side was hemodynamically insignificant (i.e., <50%). Patients with abnormal blood pressure, heart rate values, uncontrolled hyperthyroidism, renal insufficiency, hypersensitivity to iodine, or a history of adverse effects following the administration of contrast agents were also excluded from the study.

2.2 Imaging Protocol

The imaging protocol of the study included a Doppler examination, non-contrast axial computed tomography, CTP, and DSA. All

images are taken before and after the injection of an iodinate contrast medium. The DSA, as a gold standard in neuroradiology, is performed in patients with various types of vascular pathology, such as aneurysm, arteriovenous malformation, fistula, and vessel occlusion or stenosis. This radiographic technique visualizes cerebral vessels with the minimum view of a background tissues.

The CTP imaging was performed before and 3 years after ICA stenting. The imaging was done using a 64-MDct Light Speed VCT XT scanner (GE Healthcare Technologies, Waukesha, WI). Non-contrast axial computed tomography scans allowed for the identification of signs of active bleeding, acute ischemic stroke, or features of increased intracranial pressure. The axial images acquired for CT perfusion at 40 mm in cine mode (CTP 4 cm) were initiated 5 s after the start of 40 mL of the nonionic iodine-based intravascular contrast agent, OPTIRAY 350 (Mallinckrodt, St. Louis, MO), administered into an antecubital vain at an injection rate of 4 mL/s. After contrast injection, an additional bolus of 40 mL of physiological saline was administered. The acquisition parameters for the CT examinations were the following: tube voltage 80 kV, 150 mAs, slice thickness 5 mm, rotation time 1/s, field of view (FOV) 25 cm, time interval between reconstructed images 0.5–1 s, total exposure time 45 s, interval 0.5 mm, gantry angle parallel to and above the orbital roof to avoid radiation exposure to the lens, image matrix 512 x 512, and eight slices. A total of 272 slices from 34 patients were obtained, with a scan time of 45 s.

The arterial input function was chosen within the anterior cerebral artery (segment A2) on the basal ganglia level. The venous output region was selected from the superior sagittal sinus. All of the listed acquisition parameters were applied as provided by the manufacturer for the brain perfusion study.

2.3 Image Post-processing

The raw data were transferred to a dedicated diagnostic workstation (AW 4, GE Healthcare Technologies, Waukesha, WI) and analyzed using a research version of the CTP post-processing software package (CT Perfusion v4.3.1) (GE Healthcare Technologies, Waukesha, WI) to create color maps of dynamic cerebral enhancement, represented as CBF, cerebral blood volume (CBV), mean transit time (MTT), time to peak (TTP), and permeability surface area product (PS).

This diagnostic application offers two different evaluation modes. The first mode, the "neuro-brain stroke" mode, calculates the perfusion metrics of MTT, CBF, CBV, and TTP for brain stroke assessment using the maximum slope method (Lin et al. 2007; Fiorella et al. 2004). The MTT characterizes the average time of contrast agent residence within the tissue. In mathematical terms, the mean transit time is computed as the time between the initial impulse and the time of arrival. The MTT is computed and displayed in seconds. The CBF is derived from the initial value of the impulse residue function and is computed and displayed in mL *per* 100 g of wet tissue *per* minute. The CBV is computed and displayed in mL *per* 100 g of wet tissue. The blood volume is the product of blood flow and mean transit time: $CBV = CBF \times MTT$. The TTP is the time between the onset of enhancement transient (last pre-enhancement image) and the peak value of time curve (image with the maximum value before the first post-enhancement image). The time to peak is computed and displayed in seconds, using the raw time curve data directly.

The second mode, known as the "neuro-brain tumor" mode, calculates microvascular permeability (PS) and fractional blood volume based on the Johnson and Wilson model (St Lawrence and Lee 1998a, b). The PS is computed and displayed in mL *per* 100 g of wet tissue *per* minute. It is computed from the impulse residue function. Contrast agent diffusion appears in the impulse residue function as a residual enhancement that occurs after the initial impulse response and decreases exponentially with time.

Two experienced neuroradiologists blinded to clinical information (medical history, side, and time of operation) independently manually drew two standardized elliptical mirrored regions of interest (ROI). The ROI (approximately 10 cm^2 each) was determined on all analyzed levels, over

the cortical gray matter centered 20 mm from the brain edge. The large vessels were automatically excluded via brain perfusion software. The absolute values of CTP parameters (MTT, TTP, CBF, CBV, and PS) of one hemisphere in the region of middle cerebral artery distribution and contralateral mirroring areas in functional maps were measured. Then, the relative parameters were calculated as the symptomatic hemisphere-to-asymptomatic hemisphere ratios.

2.4 Patient Surveys

A survey was given to patients who qualified for the stenting procedure. The first survey was carried out on the day of admission. The second survey was performed after the follow-up perfusion scan (approximately 3 years after the procedure). The flowing questions were asked:

- Headache: Yes/No
- Dizziness: Yes/No
- Tinnitus: Yes/No
- Blurred vision: Yes/No
- Transient blindness: Yes/No
- A sense of gravity of the head: Yes/No
- Pain in the eyeballs: Yes/No

3 Results

Relative CBF, CBV, MTT, TTP, and PS values did not change 3 years after stenting, compared to the baseline values measured before stenting (Table 2). Nevertheless, a clear trend toward rMTT decline was observed. When a subgroup analysis was performed, it appeared that three separate groups of patients could be distinguished: (1) patients with rMTT decline greater than 5%, (2) patients with rMTT decline between 2% and 5%, and (3) patients with rMTT decline less than 2%. Improvement in clinical symptoms, reported by patients, was related to a decline in rMTT. A prominent improvement was reported with a rMTT decline greater than 2% (Figs. 1, 2, and 3).

In the group with the greatest rMTT decline, the number of reported headaches was reduced from 7 to 2, dizziness from 9 to 4, tinnitus from 5 to 3, blurred vision from 4 to 2, transient blindness from 2 to 1, a sense of gravity of the head from 2 to 0, and pain in the eyeballs from 3 to 1. In the group with rMTT decline between 5% and 2%, the number of reported headaches was reduced from 9 to 3, dizziness from 7 to 3, tinnitus from 11 to 4, and blurred vision and a sense of gravity of the head from 2 to 0.

Table 2 Comparison of computed tomography perfusion (CTP) at baseline before stenting and 36 after stenting

	Parametric *t*-test for dependent samples (n = 34)		
Parameter	Mean ± SD (median)	Δ change	*p*
$rMTT_1$	1.09 ± 0.27 (1.06)	−7.59%	0.155
$rMTT_3$	1.01 ± 0.20 (0.99)		
$rCBV_1$	0.96 ± 0.19 (0.96)	+6.56%	0.134
$rCBV_3$	1.02 ± 0.15 (1.03)		
$rCBF_1$	0.99 ± 0.09 (1.01)	+1.11%	0.563
$rCBF_3$	1.00 ± 0.07 (1.00)		
$rTTP_1$	1.01 ± 0.03 (1.01)	+2.08%	0.515
$rTTP_3$	1.03 ± 0.19 (1.00)		
rPS_1	1.09 ± 0.34 (1.06)	+0.74%	0.905
rPS_3	1.10 ± 0.24 (1.06)		

MTT mean transit time, *CBV* cerebral blood volume, *TTP* time to peak, and *PS* permeability surface area product; "r" stands for "relative" parameters that were calculated as symptomatic hemisphere-to-asymptomatic hemisphere ratios; "1" denotes parameters at baseline before stenting; and "3" denotes parameters 3 years after stenting procedure

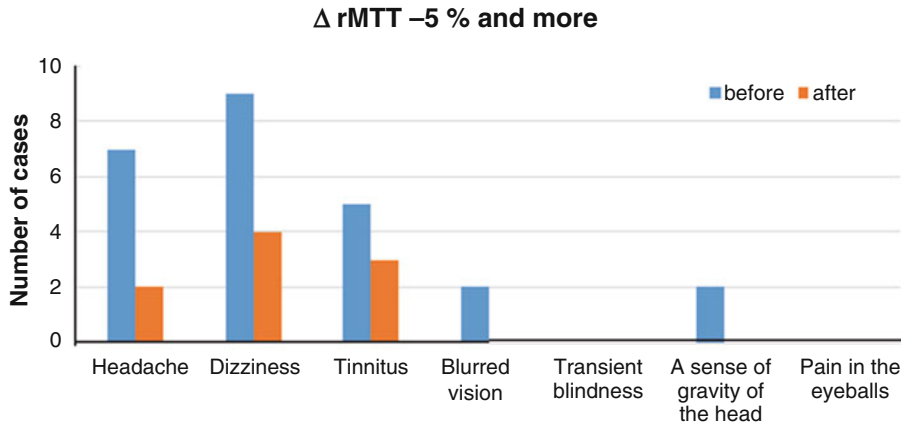

Fig. 1 Changes in clinical symptoms in patients with internal carotid artery (ICA) stenosis, associated with relative mean transit time (rMTT) decline greater than 5%, 3 years after the stenting procedure Δ

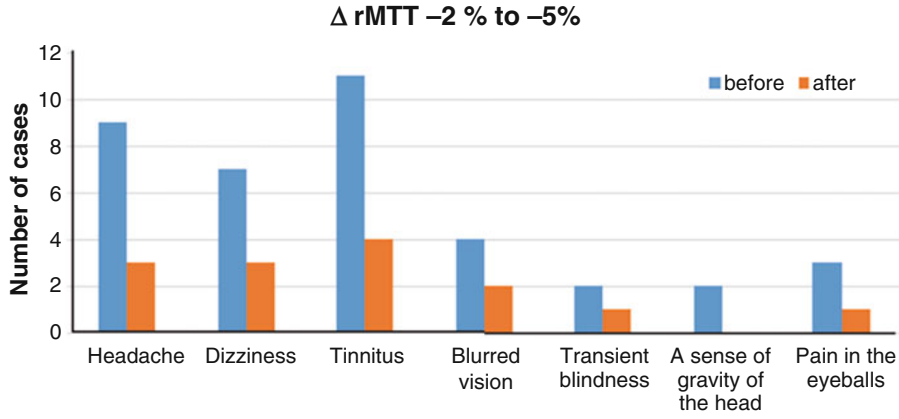

Fig. 2 Changes in clinical symptoms in patients with internal carotid artery (ICA) stenosis, associated with relative mean transit time (rMTT) decline between 2% and 5%, 3 years after the stenting procedure

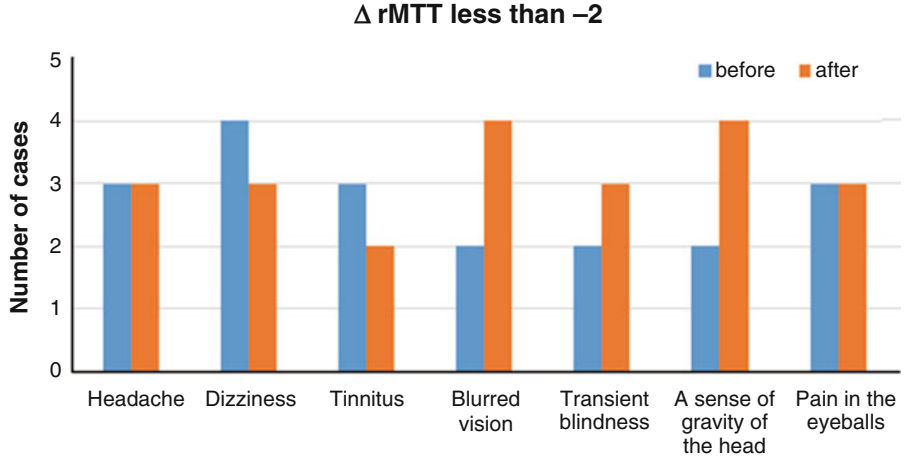

Fig. 3 Changes in clinical symptoms in patients with internal carotid artery (ICA) stenosis, associated with relative mean transit time (rMTT) decline less than 2%, 3 years after the stenting procedure

In contradistinction, in patients without an appreciable decline in rMTT (<2%), clinical symptoms either did not change or actually worsened (Fig. 3). In this group, the number of reported headaches remained unchanged (3 cases before and after ICA stenting), dizziness decreased from 4 to 3, tinnitus from 3 to 2, blurred vision increased from 2 to 4, transient blindness increased from 3 to 4, a sense of gravity of the head increased from 2 to 4, and pain in the eyeballs remained unchanged (3 cases before and after ICA stenting).

4 Discussion

The main finding of the study is that relative decreases in MTT were associated with improvements in the clinical symptoms reported by patients with ICA stenosis, 3 years after the stenting procedure, providing that the decreases were greater than 2% of the baseline level noted before stenting. Taking into account both objective measurements and self-assessed symptoms allows for a more complete evaluation of the influence of the stenting procedure. In the literature, CBF is the most often used variable for the evaluation of treatment effects in patients with ICA stenosis. There is a need to focus on the influence of treatment on the perception of quality of life, especially for the extended periods of time, longer than a year (Shan et al. 2015). The assessment of quality of life can be helpful in the estimation of the effectiveness of treatment in terms of stroke prevention and in comparison of different types of treatments, and as such it is an important therapeutic goal (Fadrná et al. 2018). Assessing quality of life in this group of patients is also vital, since the very presence of severe carotid stenosis may predispose patients to lower general well-being and lower satisfaction with life, let alone depressive and cognitive impairment (Pucite et al. 2017).

There is an ongoing discussion regarding the pathomechanisms involved in cerebral small vessel disease and the subsequent development of neurological symptoms and cognitive decline. According to some authors, vascular stiffening, increased pulse pressure, and carotid pulsatility are the major factors leading to the development of white matter hyperintensities and neurological and cognitive deterioration (Wardlaw et al. 2017; van Sloten et al. 2015; Aribisala et al. 2014; Singer et al. 2014). There is a contradicting theory, indicating that a prolonged brain hypoperfusion results in hypoxia, inflammation, and oxidative stress, followed by a blood-brain barrier collapse (Ding et al. 2017; Duncombe et al. 2017; Szarmach et al. 2017; Kim et al. 2012).

In this study we demonstrated that subjective symptoms reported by patients might be related to changes in cerebral microcirculation. Consequently, our study refocuses clinical attention on the interventional and non-interventional medical procedures aiming to maintain blood flow in cerebral small vessels, such as carotid artery recanalization, proper pharmacological therapy, and lifestyle modifications. The predominant notion of numerous reports is that MTT is the most sensitive marker of cerebral perfusion changes. However, calculation of accurate absolute values of this parameter is impractical, while using absolute threshold values in making therapeutic decisions is dubious due to the parameter's strong correlation with numerous external factors, e.g., cardiac output, age, or a degree of stenosis (Zhang et al. 2013; Esteban and Cervera 2004; Koenig et al. 2001). Consequently, we assessed relative MTT in this study, i.e., the ratio of symptomatic hemisphere-to-asymptomatic hemisphere MTT.

ICA stenosis was diagnosed in all patients by a Doppler examination more than 5 years before surgery. Due to stringent inclusion criteria, consisting of <70% stenosis within a single ICA, the population of this study was homogeneous in terms of cerebral hemodynamic parameters and was characterized by low CBF and low CBV. As a result, ICA stenosis on the contralateral side must have been hemodynamically insignificant (<50%), as evidenced by other studies (Ricotta et al. 2011; Nicolaides et al. 2005; Samson et al. 1999). Patients with bilateral hemodynamically significant, i.e., >70%, stenosis of the cranial arteries were excluded from the study. Although a relatively narrow population can be seen a limitation of this study, its

homogeneity could enhance the reliability of results. The present findings justify further research with the use of magnetic resonance imaging (MRI). In particular, a combination of dynamic susceptibility contrast MRI (DSC-MRI), dynamic contrast-enhanced MRI (DCE-MRI), and blood oxygen level-dependent imaging MRI (BOLD-MRI) could provide comprehensive information on the effect on the functional brain status of cerebral microcirculation and blood-brain barrier permeability fluctuations. Such studies with the concomitant neuropsychological assessment are currently under way.

Quantitative CTP data are highly dependent on the post-processing software. Software differences are frequently considered the main cause of variability in perfusion results relative to inter-operator and intra-operator differences (Kudo et al. 2010; Kamalian et al. 2011; Zussman et al. 2011). In this study we used the equipment manufactured by one producer and the same post-processing procedure for all subjects. Further, there were two experienced neuroradiologists who independently drew standardized elliptical mirrored ROIs, with the area of approximately 10 cm^2 each, on the section level of the reference CT image over the cortical gray matter of the middle cerebral artery territory. Finally, in all subjects, we used a coverage size of 40 mm, which is well-suited for detecting perfusion parameters owing to the high density of scans (Szarmach et al. 2016).

In conclusion, we believe we have conclusively demonstrated in this study that subjective symptoms reported by patients suffering from ICA stenosis might be related to changes in cerebral microcirculation. Further studies with the extensive use of various MRI techniques are warranted to assess how changes in the brain microcirculation and blood-brain barrier functional status are related to patients' self-reported quality of life and to overall patients' functional status.

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The experimental protocol of the study was approved by the Ethics Committee of the Medical University in Gdansk, Poland.

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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Influence of Glycemic Control on Coagulation and Lipid Metabolism in Pregnancies Complicated by Pregestational and Gestational Diabetes Mellitus

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Abstract

Hypercoagulability and altered lipid metabolism, which are observed in normal pregnancy, can be enhanced in diabetes mellitus. The aim of the study was to evaluate the influence of glycemic control on coagulation and lipid metabolism in women with pregestational (PGDM) and gestational (GDM) diabetes treated with insulin. There were 50 patients with PGDM and 101 patients with GDM enrolled into the study. Serum lipid and coagulation parameters were assessed at 18–22, 25–28, and 31–34 weeks of pregnancy and were compared within the diabetic groups with reference to the effectiveness of glycemia control. We found that poor glycemic control was associated with shortened activated partial thromboplastin time (APTT) and increased activity of antithrombin III (ATIII) in both diabetic groups and with a higher plasminogen activator inhibitor (PAI-1) content level in the GDM group. Poorly controlled PGDM was associated with higher levels of total cholesterol and high-density cholesterol (HDL) in the second trimester and triglycerides in the third trimester. In patients with poorly controlled GDM, a higher concentration of HDL was observed in third trimester, whereas a higher triglyceride level was found in both second and third trimesters. Positive correlations between total cholesterol and APTT and between triglyceride and APTT and ATIII were found in the poorly controlled PGDM group. We conclude that poor glycemic control of diabetic pregnancy impacts both lipid metabolism and the blood coagulation system.

Keywords

Coagulation system · Diabetes · Glycemic control · Lipid metabolism · Pregnancy

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1 Introduction

Increased levels of coagulation factors, enhanced thrombin generation, and suppression of

fibrinolysis are commonly found in women with uncomplicated pregnancy (Akinci et al. 2008; Comeglio et al. 1996). As pregnancy advances, changes in lipid metabolism become significant. The content of cholesterol increases by 30–60% and reaches the highest values at about 32 weeks of pregnancy. Phospholipids, especially lecithin, sphingomyelin, and cephalin, free fatty acids, triglycerides (TG), and high-density (HDL), low-density (LDL), and VLDL lipoprotein fractions, also increase (Grimes and Wild 2018). Maternal hyperlipoproteinemia observed during pregnancy helps the fetus adapt to an unfavorable diabetic environment.

Diabetes mellitus enhances activation of platelets and clotting factors (Grandl and Wolfrum 2018). Plasma coagulation activation markers, such as prothrombin activation fragment 1 + 2 and thrombin-antithrombin complexes, are elevated along with other clotting factors including fibrinogen, kallikrein, von Willebrand factor, and factors VII, VIII, XI, and XII. The fibrinolytic system is relatively inhibited in diabetes due to an increase in plasminogen activator inhibitor (PAI-1) content and the presence of abnormal clot structures that are more resistant to degradation (Kamgar et al. 2006; Vaughan 2005). Peripheral blood platelets are hyperactive in diabetes, which manifests in platelet aggregation in response to the action of platelet agonists, increased platelet contractile force, and the presence of platelet-release products (Carr 2001).

The relationship between lipid metabolism and coagulation cascade is based on the "lipid hypothesis" or "Grutzbalg hypothesis" (Yee et al. 2001). Hypertriglyceridemia is associated with factor VII activation and thus stimulation of the extrinsic coagulation pathway, which takes place through the action of a contact system. The activation of the contact system, in turn, stimulates the intrinsic coagulation pathway by acting on factors XI and IX. Factor XII undergoes self-activation owing to the contact surface created by saturated long-chain fatty acids (Lyons and Basu 2012).

Passive diffusion of glucose into endothelial cells can lead to increases in intracellular glucose concentrations, which increases oxidative stress arising from the degradation of glucose metabolites. Likewise, advanced glycosylation end products (AGE), which result from intracellular hyperglycemia, are involved with vascular damage. Further, protein glycation promotes macro- and microvascular damage. Lastly, it has been reported that hyperglycemia leads to a hypercoagulable state (Brownlee 2005; Carr 2001).

Hyperglycemia leads to the degeneration of endothelial cells and neurons triggering the following main metabolic pathways: polyol pathway, late protein glycation, activation of protein kinase C, and the hexosamine pathway (Bornfeldt and Tabas 2011; Moreno and Fuster 2004). The last two pathways increase the PAI-1 content, which promotes inhibition of fibrinolysis and enhances coagulation. Oxidative stress, developed in the course of hyperglycemia, specifically impairs endothelial functions, which associates with prothrombotic propensity (Guerin-Dubourg et al. 2017; Brownlee 2005). On the other hand, hypercoagulability and altered lipid metabolism, which are the features of normal pregnancy, can exaggerate in the presence of diabetes (Grimes and Wild 2018; Cerneca et al. 1997). In the face of the intertwined relationships among pregnancy, diabetes, oxidative stress, and the blood coagulation cascade above outlined, we set out to examine in this study the influence of glycemic control on the coagulation and lipid metabolism in women with pregestational (PGDM) and gestational (GDM) diabetes treated with insulin.

2 Methods

The PGDM group consisted of 50 patients with pregestational diabetes, which included 40 patients with White class B-D (PGDM B-D group) and 10 patients with RF class (PGDM RF group). The GDM group consisted of 101 patients, diagnosed with an oral glucose tolerance test (75 g glucose load), carried out between 24th and 28th gestational week, who required treatment with diet and insulin.

Glucose content was measured 5 times a day, and it was averaged for the 2nd and 3rd trimester of pregnancy. Glycated hemoglobin (HbA1c) and

Table 1 Glycemic control in patients with pregestational (PGDM) and gestational diabetes mellitus (GDM)

	Good glycemic control	Poor glycemic control
PGDM	15 (30)	35 (70)
GDM	73 (72)	28 (28)

Values are *n* (%) of cases in a group

Table 2 Clinical characteristics of patients with pregestational (PGDM) and gestational diabetes mellitus (GDM)

	PGDM B-D group*	PGDM R-F group*	GDM group
	$n = 40$	$n = 10$	$n = 101$
Age (years)	29.3 ± 6.25	27.0 ± 5.4	33.4 ± 6.3
Parity – multiparas (*n*)	22 (55)	4 (40)	62 (61.4)
BMI before pregnancy (kg/m^2)	24.0 ± 5.0	22.0 ± 2.0	28.3 ± 6.5
BMI before delivery (kg/m^2)	28.5 ± 4.4	27.8 ± 2.3	32.2 ± 6.0
BMI increase during pregnancy (%)	19.7 ± 10.2	26.0 ± 9.5	15.1 ± 11.0
Positive family history of diabetes mellitus (*n*)			
1st degree relatives	9 (22.5)	2 (20.0)	42 (41.6)
Other relatives	12 (30.0)	2 (20.0)	67 (66.3)
Previous diagnosis of GDM (*n*)	1 (4.5)	0	7 (11.3)
Previous diagnosis of hypertension (*n*)	3 (7.5)	3 (30.0)	16 (15.8)

Values are means ±SD or *n* (%) of cases in a group; according to the White classification of diabetes during pregnancy (Murthy et al. 2002)

fructosamine contents were measured at 6-week intervals, and they also were averaged for the 2nd and 3rd of pregnancy. The groups were divided according to effectiveness of glycemic control. Criteria for good glycemic control were as follows: mean fasting blood glucose <95 mg/dL, mean HbA1c level < 6.0%, and mean fructosamine concentration < 280 mg/dL. Distribution of patients in the groups is presented in Table 1. Clinical data, obtained from medical records, are presented in Table 2.

In the 2nd trimester, blood samples were taken twice in PGDM patients (between the 18th–22nd and 25th–28th pregnancy week) and once in GDM patients (between the 25th and 28th pregnancy weeks). In the 3rd trimester, blood samples were collected from all patients between the 31st and 34th pregnancy weeks. The following blood indices were determined to evaluate coagulation and fibrinolytic activation: number of platelets, prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen concentration, antithrombin III (ATIII) activity, globulin clot lysis time, and plasminogen activator inhibitor (PAI-1) activity. The activities of plasma ATIII and PAI-1 were determined with a coagulometric method (Bio-Ksel Sp. z o. o., Grudziądz, Poland) and with a Spectrolyse PAI-1 kit (American Diagnostica Inc., Greenwich, CT), respectively. Total cholesterol (CH-T), LDL, HDL, and TG contents were the lipid indices measured in the blood. The assessment of carbohydrate metabolism also included the mean fasting glycemia, the mean postprandial glycemia, and the diurnal glycemia in consecutive trimesters of pregnancy.

Data were presented as means ±SD. Differences between the two groups were assessed with student's *t*-test after the confirmation of normality of data distribution. Spearman's correlation coefficient was used to establish the relationship between parameters. A p-value <0.05 defined statistically significant differences.

3 Results

Glycemic control had a significant effect on the results of coagulation tests in women with PGDM and GDM in both the second and third trimesters of pregnancy (Table 3). The patients whose

Table 3 Coagulation tests in pregestational (PGDM) and gestational diabetes mellitus (GDM) patients with good and poor glycemic control

		Good glycemic control	Poor glycemic control	
PGDM		***n* = 15**	***n* = 35**	***p***
APTT (s)	2nd trimester	32.15 ± 1.98	30.03 ± 2.20	<0.010
	3rd trimester	33.27 ± 2.53	29.36 ± 4.01	<0.002
ATIII (%)	2nd trimester	117.8 ± 10.2	134.7 ± 11.5	<0.0001
	3rd trimester	128.3 ± 11.3	145.2 ± 12.8	<0.001
GDM		***n* = 73**	***n* = 28**	***p***
APTT (s)	2nd trimester	34.80 ± 2.90	32.10 ± 2.59	<0.0001
	3rd trimester	33.52 ± 3.07	29.70 ± 3.12	<0.0001
ATIII (%)	2nd trimester	107.3 ± 16.5	115.4 ± 12.8	<0.025
	3rd trimester	112.9 ± 19.1	130.8 ± 17.2	<0.0001
PAI-1 (IU/mL)	2nd trimester	15.9 ± 4.6	19.1 ± 3.2	<0.002
	3rd trimester	18.7 ± 8.1	27.3 ± 2.5	<0.0001

Values are mean+/-SD. Student's *t*-test

glycemia was poorly controlled had a shorter mean APTT and a greater activity of ATIII. In addition, patients with poorly controlled GDM presented a higher mean PAI-1 activity in both trimesters. Glycemic control had inappreciable effects on the other indices measured such as the platelet count, fibrinogen content, PT, and TT in either trimester of pregnancy in both PGDM and GDM patients.

Poorly controlled glycemia hampered lipid metabolism in pregnancy. In the PGDM group with hyperglycemia, CH-T was elevated in both trimesters, whereas the levels of HDL and TG were elevated in the 2nd or 3rd trimester in either group, respectively. In the GDM group, poor glycemic control was associated with a higher HDL content in the 3rd and a higher TG content in both the 2nd and 3rd trimester. There were no significant differences in CH-T content in this group (Table 4).

The PGDM group with poor glycemic control was selected to evaluate the correlation between lipid and coagulation indices. The analysis excluded the GDM patients, as the duration of diabetes in this group was considered too short to expect a consolidation of changes in both lipid and coagulation metabolism. Both CH-T and TG were associated with APPT, and TG was associated with ATIII activity. A particularly pronounced relationship was noticed between TG content and APTT ($r = 0.35$; $p < 0.01$) (Table 5).

4 Discussion

Pregnancy acts to increase the propensity for coagulation and impairs fibrinolysis, which is a manifestation of the adaptive mechanism to prevent excessive bleeding from the placental site during labor and postpartum (O'Riordan and Higgins 2003). Likewise, diabetes creates a hypercoagulable state due to increased activation of platelets and prothrombotic coagulation factors, accompanied with a decrease in fibrinolysis (Gorar et al. 2016; Alzahrani and Ajjan 2010). Among the proposed mechanisms of the prothrombotic influence of hyperglycemia, there are oxidative stress, along with its direct effect on gene transcription of coagulation factors, loss of the endothelial glycocalyx layer shielding the coagulation factors, and a direct glycation of coagulation factors, which alters their activity (Lemkes et al. 2010). Expectedly, co-occurrence of both conditions could potentiate these effects.

The major finding of this study was a significantly lower APTT, the essential measure of coagulability, in pregestational and gestational diabetes in patients whose hyperglycemia was poorly controlled. These results are consistent with the findings of Gorar et al. (2016) in gestational diabetes. However, literature data are contentious. van Wersch et al. (1990) have examined female and male patients with insulin-dependent

Table 4 Lipid profiles in pregestational (PGDM) and gestational diabetes mellitus (GDM) patients with good and poor glycemic control

		Good glycemic control	Poor glycemic control	
PGDM		***n* = 15**	***n* = 35**	***p***
CH-T (mg/dL)	2nd trimester	210.9 ± 19.5	240.8 ± 18.1	<0.0001
	3rd trimester	225.3 ± 28.7	307.2 ± 14.6	<0.0001
HDL (mg/dL)	2nd trimester	54.9 ± 12.1	60.8 ± 5.7	<0.0300
	3rd trimester	71.4 ± 16.9	61.4 ± 23.6	ns
Triglycerides (mg/dL)	2nd trimester	255.9 ± 72.4	28.3 ± 92.0	ns
	3rd trimester	274.4 ± 68.1	352.8 ± 74.5	<0.002
GDM		***n* = 73**	***n* = 28**	***p***
HDL (mg/dL)	2nd trimester	55.1 ± 12.3	57.9 ± 0.8	ns
	3rd trimester	56.1 ± 18.2	78.4 ± 7.9	<0.0001
TG (mg/dL)	2nd trimester	178.3 ± 46.5	214.5 ± 89.5	<0.0100
	3rd trimester	225.9 ± 53.1	312.7 ± 68.4	<0.0001

Values are means ±SD. Student's *t*-test
CH-T total cholesterol, *HDL* high-density lipoprotein, *TG* triglycerides, *ns* nonsignificant

Table 5 Correlation between lipids and coagulation tests in pregestational diabetes mellitus (PGDM) patients with poor glycemic control

	APTT		**ATIII**	
	r	*p*	*r*	*p*
CH-T	0.27	<0.04	ns	ns
TG	0.35	<0.01	0.27	<0.03

APTT activated partial thromboplastin time, *ATII* antithrombin III, *CH-T* total cholesterol, *TG* triglycerides. Spearman's rank correlation coefficient test; *ns* nonsignificant

diabetes and have noticed a prolongation of APTT in subjects with lower HbA1 values.

Hyperglycemia reduces the action of antithrombin III, a natural factor X inhibitor (Erem et al. 2005), which decreases the formation of thrombin-antithrombin complexes, and thus also thrombin overactivity, and enhances propensity for hypercoagulability. There are data that demonstrate either a positive (Griffin et al. 2001) or negative (Ceriello 1993) relationship between HbA1 and ATIII activity. In this study, ATIII activity was significantly higher in both 2nd and 3rd trimesters of pregnancy in PGDM and GDM patients with unsatisfactory glycemic control. This result is consistent with the observations of Donders et al. (1993) who have found a positive correlation between the content of glycated hemoglobin and ATIII activity in patients with diabetes. Such a relationship may be a manifestation of a defense mechanism against endovascular coagulation or a reflection of increased ATIII release from damaged endothelium. ATIII activity may also be reduced due to nonenzymatic glycation (Ceriello 1993). Decreased antithrombin activity has been noticed in nonpregnant patients with insulin-dependent diabetes, irrespective of the incidence of vascular complications, in a study of Leurs et al. (1997). There are no data in the literature evaluating this aspect in pregnant women.

In pregnancy, a decrease in fibrinolysis and enhancement of coagulation also occur due to an increase in PAI-1 and PAI-2 that are synthesized by the endothelium and placenta, respectively. High plasma levels of PAI-1 have been shown in normal pregnancy (Cerneca et al. 1997). Kvasnicka et al. (1996) have reported elevated PAI-1 content also in gestational diabetes. Hyperexpression of the PAI-1 antigen is present in nonpregnant patients with type 1 and type 2 diabetes (Vaughan 2005). Poorly controlled diabetes can decrease fibrinolysis by reducing the nonenzymatic glycosylated susceptibility to plasmin digestion. Untreated fibrin deposits accumulate in tissues and contribute to the development of diabetic complications. The available knowledge can hardly enable the presentation of a unified and unambiguous view on the function of fibrinolysis in patients with diabetes. Both a

decrease (Kearney et al. 2017; Carr 2001) and no change (Gorar et al. 2016; Bellart et al. 1998) in fibrinolytic activity have been noticed. The discrepancy may result from inhomogeneity of patients investigated concerning the type and duration of diabetes, the effectiveness of glycemic control, and the coexistence of complications. In our study, PAI-1 activity was significantly enhanced in the condition of poor glycemic control in GDM patients only. This finding is consistent with those of Kvasnicka et al. (1996) in GDM and Erem et al. (2005) in non-insulin-dependent diabetes.

A decrease in fibrinolysis often associates with disorders of lipid metabolism and diabetes (Latron et al. 1991). Also, a relationship between PAI-1 content and serum CH-T and TG has been demonstrated. However, no effect of insulin has been noticed on PAI-1 in diabetic patients, irrespective of the presence of vascular complications. Konieczynska et al. (2014) have shown that PAI-1 content is unaffected by glycemic control and diabetes duration in patients with non-insulin-dependent diabetes. In contradistinction, in the present study, we showed that poor glycemic control increased PAI-1 activity in GDM patients. It seems that hyperglycemia and hypoinsulinemia are responsible for abnormal lipid metabolism (Ritchie et al. 2017; Briguori et al. 2004). In patients with insulin deficiency, reduced lipoprotein lipase activity impedes the removal of triglyceride-rich lipoproteins from circulation. Cholesterol is essential for normal fetal development, as it is essential for the formation of cell membranes. In the present study, patients with unsatisfactorily controlled PGDM had a higher level of CH-T, and those with GDM had a higher level of TG in the 2nd and 3rd trimesters of pregnancy. That is consistent with the studies in which glycemic control, assessed by HbA1c, correlated with the contents of CH-T, LDL, and triglycerides (Koukkou et al. 1996). We also found that patients with poorly controlled PGDM had a significantly higher mean HDL content in the 2nd trimester. However, diabetic-induced changes in HDL are less clear. In the DCCT study, a lower HDL level and a higher TG level, associated with the effectiveness of diabetes control, assessed from the value of HbA1c, were found in young women with insulin-dependent diabetes (DCCT Research Group 1992).

Hypertriglyceridemia is associated with high levels of prothrombin, fibrinogen, and factors VII, VIII, IX, X, and PAI-1 (Griffin et al. 2001). In the present study, however, we noticed positive associations only between ATIII activity and a shortened APTT and hypertriglyceridemia in patients with poorly controlled PGDM. In a study of Erem et al. (2005), ATIII activity was associated with plasma CH-T and TG. Although the plasma lipids associate with the severity of diabetes mellitus, the level of glycemia, above which disorders of lipid metabolism appear, is unknown (Kalaria et al. 2016). The exact mechanisms that underlie the influence of hyperlipidemia on fibrinolytic activity and in particular on PAI-1 activity are still elusive (Morelli et al. 2017). Any factors that change the balance between thrombin generation and fibrinolytic activity may lead to the formation of thrombosis or bleeding complications.

The regulation of hemostasis and thrombosis involves numerous plasma factors that contribute to procoagulant and anticoagulant pathways. Lipids and hyperglycemia are among such factors. Procoagulant lipids/lipoproteins include triglyceride-rich particles and oxidized low-density lipoprotein (LDL) in plasma which can accelerate the activation of prothrombin and factors VII and X. The potentially anticoagulant lipids and lipoproteins, including HDL, enhance the inactivation of factor Va (Griffin et al. 2001). The procoagulant and anticoagulant lipoproteins in the plasma are a viable part of the regulatory system of thrombin generation.

In conclusion, poor glycemic control adversely affects lipid metabolism and coagulation process in pregnancies complicated by diabetes. Hampered lipid metabolism intensifies the prothrombotic propensity and decreases fibrinolytic activity.

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of Warsaw Medical University in Warsaw, Poland.

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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Maternal Nutritional and Water Homeostasis as a Presage of Fetal Birth Weight

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Abstract

Birth weight is a key determinant of perinatal outcomes which affect physical development and metabolic function. In this study, we evaluated the potential role of maternal body composition and nutritional status in programing fetal birth weight. This was a longitudinal study that included 29 pregnant women and their full-term newborns. Maternal dietary energy and fluid intake and body adipose tissue were assessed. In addition, we measured the serum content of copeptin, aldosterone, and angiotensin II in maternal and umbilical cord blood. The measurements were done across the three trimesters of pregnancy, on average, at 11.6 weeks, 18.3 weeks, and 30.2 weeks. Each newborn's birth weight was determined at the percentile line, using the World Health Organization (WHO) standards based on the gestational age, gender, and weight. We found no appreciable relation of fetal birth weight to the maternal dietary and fluid intakes, and the content of angiotensin II, aldosterone, or copeptin. However, birth weight correlated with increases in body adipose tissue in early pregnancy stages. Further, birth weight correlated positively with copeptin and adversely with angiotensin II in cord blood. We conclude that the present findings may be helpful in the assessment of a critical level of body adipose tissue in women of child-bearing age, above which the potential risk of macrosomia appears. The female population of child-bearing age needs a continual update on the nutritional knowledge to prevent modifiable maternal and fetal perinatal complications.

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Keywords
Angiotensin · Birth weight · Body adipose tissue · Body composition · Copeptin · Newborns · Nutrition · Pregnancy · Umbilical cord blood · Water homeostasis

1 Introduction

At the time of rapid development, such as an embryonic or fetal life, a number of organ structures and associated functions undergo programing, which determines the set point of physiological and metabolic responses to factors that carry into adulthood (Stout et al. 2015; Barker 1998). The environment "in utero" has been broadly studied in the framework of the Developmental Origins of Health and Disease (DOHaD) approach that evolved from epidemiological studies of infant mortality. According to the DOHaD hypothesis, increased susceptibility to diseases is partly shaped during fetal programing by early-life exposures through maternal diet, lifestyle, and other environmental conditions (Guéant et al. 2013). Nutritional status of the mother, which is an important factor that affects the programing of the body, involves factors such as maternal body composition, dietary and fluid intake, blood flow to the uterus and placenta, and fetal genes (Kwon and Kim 2017).

One of the major components of DOHaD is birth weight. Size at birth is an indicator of exposure to prenatal perturbations, which affect physical development and metabolic function. On one hand, evidence from epidemiological studies indicates that small size at birth is a risk factor for a range of metabolic problems, including high adult body mass index (BMI), insulin resistance, increased visceral adiposity, and impaired glucose tolerance (Stout et al. 2015; Calkins and Devaskar 2011). On the other hand, fetal macrosomia increases the risk of perinatal morbidity, mortality, and adverse developmental outcomes, especially obesity-related metabolic disorders later in life (Briana et al. 2017). Moreover, maternal obesity has been associated with increased risk of fetal macrosomia, neonatal adiposity, and metabolic syndrome in progeny (O'Connor et al. 2014). Thus, maternal body mass and body composition are modifiable risk factors of fetal macrosomia.

Several human studies have shown that the total body water accretion during pregnancy is associated with birth weight and it is considered a predictor of fetal well-being (Most et al. 2018; Larciprete et al. 2003). However, a direct relationship between total fluid intake, water intake, or intake of any other fluid type and pregnancy outcome and birth weight are rarely investigated. Experimental studies indicate that increased activity of the systems that play an essential role in the salt and water homeostasis, i.e., the sympathetic and renin-angiotensin-aldosterone pathways, and also enhanced oxidative stress or endothelin level has a substantial influence on the developmental programing of blood pressure in later life. A shift in the redox status toward the pro-oxidative side is observed in low-birth-weight humans and in the experimental models of developmental insult. The importance of oxidative stress and endothelin as mediators of increased cardiovascular risk is also demonstrated in animal studies (Alexander et al. 2015). In view of a paucity of data on factors shaping birth weight, this study seeks to define the potential role of maternal nutrition and water homeostasis in birth weight programing. To this end, maternal body composition, diet, and the serum content of copeptin, aldosterone, and angiotensin II were longitudinally assessed in pregnant women.

2 Methods

2.1 Study Population and Protocol

Seventy pregnant women up to the 12th week of pregnancy were enrolled into this study, conducted at the First Department of Obstetrics and Gynecology and the Department of Social Medicine and Public Health of Warsaw Medical University in Warsaw, Poland, from October 2014 to March 2017. All the patients were Caucasians and represented a college/university education level. Exclusion criteria were:

pre-pregnancy diabetes and hypertension, and heart, kidneys, liver, or endocrine disorders. The study was of a longitudinal nature. Patients were assessed in the 1st, 2nd, and 3rd trimester of pregnancy, i.e., up to week 12 and between week 15–week 20 and week 27–week 32 of gestation, respectively. In detail, the assessments were made, on average, at 11.6 week, 18.3 week, and 30.2 week. Out of the 70 pregnant women recruited for the study, 41 were lost to follow-up. Therefore, the final sample consisted of 29 pregnant women and 29 newborns.

At each visit, patients' clinical and demographic data were collected by means of a questionnaire. Anthropometric measures were taken and the diet was assessed. Skin-fold thicknesses was measured in triplicate on the right body side with a caliper (Holtain Ltd., Crosswell, UK) and the percentage of body fat (%BF) was calculated with the Yuhasz Body Density Equation (Shephard 1991). The newborns' weight was assessed at birth. The assessment of the diet was based on 7-day records of food consumption, which was checked by trained investigators during face-to-face interviews. Food consumption data was converted into energy and nutrient intake using the current "Polish Food Composition Tables" and "Diet 5" analysis software of the National Food and Nutrition Institute in Warsaw, Poland. Maternal fasting blood samples were taken twice in the 1st and 3rd trimesters. Mixed arteriovenous blood was taken in the newborns from the umbilical cord into pyrogen-free tubes. The blood was immediately centrifuged and the supernatant was frozen to −80 °C until further use. The plasma protein content was measured using the enzyme-linked immunosorbent assay (ELISA) kits for human angiotensin II (Cat. No. EKE-002-12), human copeptin (both purchased from Phoenix Pharmaceuticals Inc.; Burlingame, CA), and human aldosterone (ref. DE5298; Demeditec Diagnostics GmbH, Kiel, Germany), according to manufacturer's instructions. Sensitivities of the tests were 0.09 ng/mL, 0.12 ng/mL, and < 5.7 pg/mL, respectively.

Other data concerning the newborns consisted of gender, birth weight, body length, head circumferences, and the Apgar score. Normal body weight was considered as the 2500–4000 g range, with microsomia and macrosomia below and above those limits, respectively. Each newborn's birth weight was determined at the percentile line, using the World Health Organization (WHO) standards based on the gestational age, gender, and weight. Ponderal index (PI), a measure of leanness (corpulence) of a newborn calculated as a relationship between mass and height, was calculated. Additionally, small for gestational age (SGA), appropriate for gestational age (AGA), and large for gestational age (LGA) categories were defined according to international standards based on the Intergrowth-21 Project that complemented the WHO standards (Villar et al. 2014).

2.2 Data Elaboration

Qualitative data were expressed as means ±SD and categorical data as counts and percentages. The Shapiro-Wilk test was used to assess data distribution and the Breusch and Pagan test to assess the possible nonlinear forms of heteroscedasticity. One-way ANOVA for repeated measures was used to assess differences in the energy and fluid intake, sodium, maternal fat, and blood content of biochemical indices measured across the three trimesters of pregnancy. Linear regression models were created to assess changes in the newborn's birth weight at the percentile line depending on the contents of copeptin and angiotensin in cord blood, and on the mother's adipose tissue content during the 1st trimester and the mother's body mass gain between the 1st and the 3rd trimester of pregnancy. The Wald test, with a significance set at $\alpha = 0.01$, was used to assess the significance of the explanatory variables in these models. Otherwise, a p-value <0.05 defined statistically significant differences. The analyses were performed using the R Statistical free software, IBM SPSS statistics v20 (IBM; Armonk, NY), Epi Info v7.2 – a free statistical package created by the Centers for Disease Control and Prevention (CDC, Atlanta, GA), and Microsoft Excel (Redmond, WA).

3 Results

The mean age of pregnant patients was 31.9 ± 3.9 years and the 1st trimester's BMI was 22.9 ± 2.6 kg/m^2. There were two cases of gestational diabetes diagnosed. Concerning the newborns, there were three macrocosmic ones, one microsomic, and another four were born preterm. Detailed clinical features of mothers and newborns are presented in Table 1.

3.1 Energy, Fluid, and Sodium Intakes and Maternal Adipose Tissue and Body Mass Changes Across Pregnancy

There were no appreciable differences noticed in the maternal energy, fluid and sodium intakes, and the percentage of adipose tissue across pregnancy trimesters (Table 2). In each trimester, a majority of patients reported sodium ingestion that was above the tolerable upper intake level (UL). About one half of patients gained more than 2.5 kg between the 1st and the 2nd trimester, with two pregnant women gaining > 7 kg. The analysis of a relationship between maternal micronutrients, macronutrients, and fluid intakes showed no association with birth weight of the progeny and with the maternal serum content of aldosterone, copeptin, and angiotensin II across the trimesters, nor with the cord blood content of these variables.

3.2 Content of Aldosterone, Angiotensin II, and Copeptin Content

The content of maternal aldosterone significantly increased in the 3rd trimester compared to the 1st trimester ($p < 0.01$). Interestingly, aldosterone in cord blood was approximately ten times higher than that in the maternal blood in the 3rd trimester. However, large discrepancies in the level of aldosterone were observed among the newborns. There were no significant differences between the maternal content of angiotensin II and copeptin across the trimesters. The cord blood aldosterone, angiotensin II, and copeptin did not differ between the newborns delivered vaginally or by C-section (Table 3).

3.3 Birth Weight and Umbilical Cord Blood Copeptin and Angiotensin II Content

To assess the relationship between different variables and either birth weight or birth weight percentile, several linear regression models were created. Two of them were statistically significant at an alpha level of 0.05, evaluated by the Wald test. There were no problems with multicollinearity of explanatory variables; the variance inflation factor (VIF) was <3. The error term was normal and homoscedastic and associations were statistically significant at a significance level < 0.05.

The first model explains the variability of birth weight with changes in the percentage of maternal body adipose tissue during the 1st trimester of pregnancy and with the umbilical cord blood angiotensin II content (Table 4). The birth weight was higher by 49.2 g per each 1% increase in body adipose tissue in women during the 1st trimester of pregnancy, with the other variables being constant in the model. Concomitantly, birth weight was lower by 177.7 g per each 0.1 ng/mL increase of cord blood angiotensin II content.

The second model explains the variability of birth weight percentile with changes in the percentage of maternal body adipose tissue during the 1st trimester of pregnancy, maternal body mass gain between the 1st and the 3rd trimester of pregnancy, and in the content of copeptin in cord blood (Table 4). The birth weight was higher by 2.6 percentile points per each 1% increase in body adipose tissue in women during the 1st trimester of pregnancy, with other variables constant in this model. Further, birth weight was higher by 3.3 percentile points per each 1 kg gain in mothers' body mass between the 1st and the 3rd trimester of pregnancy. Concomitantly,

Table 1 Clinical characteristics of mothers ($n = 29$) and newborns ($n = 29$)

Variables	Means ± SD or *n* (%)
Maternal age (years)	31.9 ± 3.9
Smoking before pregnancy	
Yes	7 (24.1)
No	22 (75.9)
Parity	
First	15 (51.7)
Other	14 (48.3)
Ethnicity	
Caucasian	29 (100)
Education	
Higher education	29 (100)
1st trimester BMI[a]	22.9 ± 2.6
Mode of delivery	
Vaginal	21 (72.5)
Cesarean section	8 (27.5)
Gender of newborns	
Male	19 (65.5)
Female	12 (41.5)
Gestational age at birth (weeks)	39.0 ± 1.5
Birth weight (g)	3511 ± 489.6
Percentiles for birth weight by gestational age	67.8 ± 28.9
SGA	1 (3.4)
AGA	20 (69.0)
LGA	8 (27.6)
PI	2.07 ± 1.05

Categories of gestational age: *SGA* small gestational age, *AGA* appropriate gestational age, *LGA* large gestational age, *PI* Ponderal index

[a]Data concerning prepregnancy body mass unavailable

birth weight was higher by 13.6 percentile points per each 1 ng/mL increase of cord blood copeptin.

4 Discussion

Meeting the basic nutritional requirements, maintaining optimal gestational weight gain and maternal body composition by pregnant women are essential for health and well-being of both mother and child. This study aimed to investigate the maternal dietary energy and fluid intake and the serum content of aldosterone, angiotensin II, and copeptin across pregnancy trimesters in relation to birth weight of the progeny. Such an investigation seems rational to specify factors that could have a substantially influence on birth weight and, in turn, for the prevention of maternal and fetal complications.

The findings of the study failed to substantiate the presence of any appreciable associations between maternal diet or fluid intake, on the one side, and newborn's birth weight and the maternal or umbilical cord blood content of aldosterone, angiotensin II, and copeptin, on the other side, across the pregnancy trimesters. This result runs against a common recommendation for pregnant women to increase energy intake as the pregnancy progresses, especially in the 2nd and 3rd trimesters. In line with the present results, however, some previous studies have also failed to show the fulfilment of the recommendation, as longitudinal changes in pregnant women's caloric intake are unnoticeable (Abeysekera et al. 2016). Savard et al. (2018) have reported that dietary intakes are below the Canadian recommendations in 36.7% of the 79 pregnant women in the 1st, 63.3% in the 2nd, and 70.9% in the 3rd trimester

Table 2 Trimester-specific maternal energy, fluid, sodium intakes, and the body adipose tissue (BAT) ($n = 26$)

	1st trimester			2nd trimester			3rd trimester			
		%Below EER, NV, AI, or UL	%Above EER, NV, AI, or UL		%Below EER, NV, AI, or UL	%Above EER, NV, AI, or UL		%Below EER, NV, AI, or UL	%Above EER, NV, AI, or UL	*p*
Energy intake (kcal/day)										
EER	1800–2050	–	–	2160–2410	–	–	2275–2525	–	–	
Mean ± SD	1855 ± 341	34.6	30.8	1805 ± 326	84.7	0	1856 ± 351	96.2	3.8	0.82
Median	1883	–	–	1885	–	–	1878	–	–	
Maternal BAT (%)										
NV	17–28	–	–	17–28	–		17–28	–	–	
Mean ± SD	24.2 ± 6.6	11.5	15.4	24.9 ± 5.9	7.7	38.5	27.1 ± 6.2	0	46.2	0.18
Median	22.1	–	–	24.5	–	–	25.9	–	–	
Fluid intake (ml/day)										
AI	2300	–	–	2300	–	–	2300	–	–	
Mean ± SD	2168 ± 577	61.6	38.4	2447 ± 755	50.0	50.0	2621 ± 743	26.9	74.1	0.07
Median	2066	–	–	2296	–	–	2605	–	–	
Sodium intake (mg/day)										
UL	2300	–	–	2300	–	–	2300	–	–	
Mean ± SD	3076 ± 791	19.2	80.8	3055 ± 578	7.7	92.3	3179 ± 782	7.7	92.3	0.80
Median	2998	–	–	3022	–	–	3101	–	–	

EER estimated energy requirement, consistent with Polish Institute of Food and Nutrition recommendations, to which an additional 360 or 475 kcal were added in the 2nd and 3rd trimesters; *AI* adequate intake, *NV* normal value, *UL* tolerable upper intake level. "–" depicts the lack of an established dietary reference intake value for a variable. Statistical evaluation performed with one-way ANOVA test for repeated measures

Table 3 Serum content of aldosterone, angiotensin II, and copeptin in maternal blood in the 1st and 3rd trimester of pregnancy and in cord blood

	1st trimester	3rd trimester	*p*	Cord blood
Aldosterone (pg/mL)				
Mean ± SD	374.0 ± 233.6	592.1 ± 157.9	<0.01	6216.2 ± 10,429.1
Median	332.4	578.8		2882.7
	($n = 28$)	($n = 28$)		($n = 24$)
Angiotensin II (ng/mL)				
Mean ± SD	0.36 ± 0.21	0.29 ± 0.17	0.18	0.22 ± 0.14
Median	0.40	0.25		0.17
	($n = 28$)	($n = 29$)		($n = 26$)
Copeptin (ng/mL)				
Mean ± SD	0.45 ± 0.26	0.56 ± 0.61	0.40	0.95 ± 0.81
Median	0.38	0.36		0.67
	($n = 28$)	($n = 29$)		($n = 26$)

Table 4 Relationship between birth weight expressed in grams (Model 1) or birth weight percentiles expressed in points (Model 2) and the analyzed variables

Model	Variable	Estimate	*p*
1	Maternal % of body adipose tissue during 1st trimester	49.2	0.017
	Cord blood angiotensin II	−177.7	0.019
2	Maternal % of body adipose tissue during 1st trimester	2.6	0.011
	Maternal body mass gain between 1st and 3rd trimester of pregnancy	3.3	0.069
	Cord blood copeptin	13.6	0.029

of pregnancy. In this study, these figures amounted to 33.3%, 84.7%, and 96.3% of the 29 pregnant women investigated in the respective trimesters of pregnancy.

Maternal weight gain during pregnancy is considered a key determinant of perinatal outcome. The guidelines of the Institute of Medicine (US) to optimize maternal, fetal, and infant health outcomes differentiate the gestational weight gain recommendations according to the prepregnancy BMI value. These guidelines also advocate that women achieve healthy body weight before pregnancy (Institute of Medicine US and National Research Council US 2009). Hulmán et al. (2015), using both quantile and linear regression statistical approaches, have reported that the gestational weight gain correlates with infant birth weight. In light of this report, the authors promote a strategy to mitigate the gestational weight gain on the population basis. However, O'Higgins et al. (2018) in a study encompassing 552 pregnant women have pointed out that when birth weight is subtracted from total gestational weight gain, the gestational weight gain loses the correlation with birth weight. Hence, a positive correlation between the gestational weight gain and birth weight may be accounted for by an antenatal contribution of fetal weight to gestational weight gain. In this study, there were only two women who displayed an excessive weight gain between the 1st and the 3rd trimesters.

Body adipose tissue can accurately reflect the maternal body composition and is considered a better predictor of birth weight than BMI. There are reports showing that maternal body adipose tissue is a major determinant of birth weight (Wang et al. 2017; Toro-Ramos et al. 2016; O'Connor et al. 2014). In line with those reports, we found in this study that the percentage of maternal adipose tissue in early stages of pregnancy has a relation to birth weight. Notwithstanding the numerous studies and meta-analysis made on the subject, no consensus has ever been reached regarding the optimal gestational weight gain for different maternal BMI categories (Robillard et al. 2018). Yet, prevention of

overweight and obesity in women of child-bearing age seems a reasonable approach. Findings from the Helsinki Birth Cohort Study show that a higher maternal BMI associates with less favorable body composition in the offspring (Eriksson et al. 2015). It is accepted that both body weight and composition in women of child-bearing age are essential factors in the DOHaD hypothesis.

Hydration during pregnancy is another such factor. In women with normal amniotic fluid volume, oral hydration increases the amniotic fluid index by approximately 16%, whereas fluid restriction decreases this index by 8% (Mulyani et al. 2017). In this study, we did not confirm the presence of a relation between fluid intake and birth weight, serum angiotensin, aldosterone, and copeptin. However, the women of this study failed to follow the fluid intake recommended for each trimester of pregnancy. Fluid intake was below that recommended in 61.6% of women in the 1st, 50.0% the in 2nd, and 26.9% in the 3rd trimesters. Bardosono et al. (2016) have also reported that 42% out of the 300 pregnant Indonesian women fail to reach the adequate intake of water. In light of these observations, it seems important to promote adequate water intake during pregnancy, especially in its early stages. This issue needs to be further explored in studies focusing on amniotic fluid index and body water balance during pregnancy.

The renin-angiotensin-aldosterone system (RAAS) plays an essential role in the salt and water homeostasis. Angiotensin II is the most biologically active peptide in the system and exerts its effects on sodium reabsorption and vasoconstriction. Data on the effects on the in utero fetal development of angiotensin II are scarce during pregnancy (Svitok et al. 2017). In this study, there was no appreciable relation between salt and water intake across pregnancy trimesters, on the one side, and the maternal serum angiotensin II and aldosterone content, on the other side. We found, however, that increasing cord blood angiotensin II correlated with decreasing birth weight. This finding is somehow in line with an experimental study that has shown that the kidney tissue angiotensin II content is significantly higher in the intrauterine growth-restricted rats than that in healthy rats (Chou et al. 2008). Other studies have also reported the increased activity of plasma renin, angiotensin I, and angiotensin II in low-birth-weight lambs (Wang et al. 2015). The suggestion arises that small size at birth is associated with increased risk of adult cardiovascular disease in later life and this association may partly be a consequence of early disordered programing of the RAAS.

This study shows that maternal plasma aldosterone was significantly higher in the 3rd trimester compared to the 1st trimester of pregnancy, and it was much higher in the cord blood compared to maternal blood. These results are in line with those of some previous studies in which the increased aldosterone over the pregnancy course coincides with increasing sodium retention (Martinerie et al. 2009; Beitins et al. 1972). This explanation seems a viable plausibility in view of sodium intake much above the tolerable upper limit in the majority of our patients (Table 2), although we did not directly assess the magnitude of sodium retention. The increase in aldosterone may be related to the activation of the RAAS system in pregnancy, which is strongly expressed in case of sodium retention. There are other possible explanations of hyperaldosteronism during pregnancy, such as increased plasm content of progesterone and estradiol and thus increased excretion of these hormones, causing antagonism to aldosterone excretion at the level of renal tubular system (Katz and Kappas 1967).

This study also shows a markedly higher level of aldosterone in cord blood compared to maternal blood. That confirms older findings showing that fetal adrenals are capable of synthesizing and secreting aldosterone, as of week 15 gestation, independently of a small portion of it crossing the placenta (Bayard et al. 1970; Dufau and Villee 1969). Further, Martinerie et al. (2009) have shown that healthy newborn infants exhibit partial resistance to aldosterone with high plasma levels of aldosterone and renin. In contradistinction, Traversa et al. (2018) have reported that compared to the maternal blood, cord blood contains less aldosterone, cortisol, and androstenedione, accompanied by a greater content of

upstream precursors, which suggests that birth involves a limiting step in the adrenal steroid biosynthesis.

Arginine vasopressin is yet another key player in the water homeostasis (Bardosono et al. 2016). The measurement of vasopressin is rather difficult and it is subject to considerable preanalytical errors due to the hormone's short half-life and instability in the serum. Copeptin, a stable C-terminal fragment of pre-provasopressin, is considered a stable surrogate for vasopressin as a potential stress marker in newborns, reflecting also a degree of hydration, in the early adaptation period to life (Jarosz-Lesz and Maruniak–Chudek 2015; Morgenthaler et al. 2008). Plasma copeptin content increases in response to increased osmolality and dehydration and thus is of relevance in regulating fluid balance and vascular tone (Benzing et al. 2011). In this study, there was no relation between copeptin content and maternal water intake. However, given the significance of glucose and insulin in fetal growth and the fundamental role of copeptin in insulin metabolism, it is reasonable to assume that the hormone plays a regulatory role in excessive fetal growth (Lukaszyk and Malyszko 2015). This study indeed shows that copeptin in cord blood correlated with birth weight, which is in line with the known relation between vasopressin release and increased adipose tissue deposition (Briana et al. 2016).

Koch et al. (2011) have assessed the content of various vasoactive and natriuretic mediators, including copeptin, in the blood of healthy adults and in cord blood. In that study, the content of copeptin is significantly higher in cord blood. Further, those authors have reported that cord blood copeptin was substantially elevated in case of vaginal delivery versus elective C-section, exceeding even the values described in the population of critically ill adult patients. The present findings were partly in line with that study, showing that cord blood copeptin was about twice as high as that in the maternal blood. In contradistinction, we found that the content of cord blood copeptin did not depend on the way of baby delivery.

A limitation of this study is a small population size. Also, an observational design of the study precluded the establishment of causality. However, we believe the study was warranted in the face of a paucity of information on the factors underlying fetal weight and the contentiousness of existing literature results on the subject. In synopsis, fetal birth weight associates with the maternal body adipose tissue during the 1st trimester of pregnancy. Further, birth weight correlates positively with copeptin and adversely with angiotensin II in cord blood. The clinical relevance of these observations is not entirely clear and should be explored using alternative study designs. We conclude that the present findings may be helpful in the assessment of a critical level of body adipose tissue in women of child-bearing age, above which there would appear a potentially modifiable risk of macrosomia. The female population of child-bearing age should be kept updated with the continually progressing nutritional savvy to prevent maternal and fetal perinatal complications.

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by an institutional Ethics Committee.

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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Cytotoxicity, Oxidative Stress, and Autophagy in Human Alveolar Epithelial Cell Line (A549 Cells) Exposed to Standardized Urban Dust

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Abstract

Exposure to urban airborne particulate matter (PM) associates with adverse health effects, but the exact mechanisms remain unclear. In this study, we focused on cytotoxicity (MTT), oxidative stress (DCF/FC), DNA damage (PI/FC), necrosis/apoptosis (FC), and autophagy (LC3 expression; WB/FC) triggered by urban dust (UD) in naïve human alveolar epithelial A549 cells and in the cells with reduced glutathione (GSH). The A549 cells were grown in F12K/FCS media supplemented with coarse carbon black (CB; Huber990; 260 nm diameter; 200 $\mu g \cdot ml^{-1}$) or urban dust (UD; Standard Reference Materials; 200 $\mu g \cdot ml^{-1}$) for 24 h. To deplete intracellular glutathione (GSH), l-buthionine-(S,R)-sulfoximine (BSO; 100 mM; 24 h) was used. Pre-treatment with BSO depleted the cellular GSH by about 30%. A similar effect was noticed after UD. The CB was without any effects on the parameters tested, except for LC3 expression (autophagy) which increased by about twofold. However, UD decreased cell viability by about 27%, decreased cell proliferation in BSO pre-treated cells, increased ROS production, and increased both Hsp70 and LC3 proteins by about twofold, but most changes were unrelated to ROS-mediated GSH depletion. We conclude that urban dust-induced oxidative stress is important in PM toxicity, but other as yet unrecognized mechanisms are also involved.

Keywords

A549 cells · Alveolar cells · Apoptosis · Autophagy · Cytotoxicity · Oxidative stress · Particulate matter · Urban dust

1 Introduction

Exposure to air pollutants causes many health problems including asthma, COPD, cardiopulmonary diseases, lung cancer, and birth defects (Li et al. 2019; Ali et al. 2018). The consequences of exposure are highly unpredictable, but the exposure brings about persistent inflammation of the airways (Hankey and Marshall 2017; Hüls et al. 2017). Short-term exposures to air particulate matter (PM) usually exacerbate pre-existing diseases, especially in respiratory and cardiovascular system, depending on the individual age and health status, and increase hospital admissions. Long-term exposures, on the other side, increase

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the rate of disease progression and significantly reduce life expectancy (Wong et al. 2016). Due to high inconsistency of clinical and experimental data in this field, it is crucial to establish some biomarkers for PM exposure, which may unravel the complexity of relationship between exposure and respiratory health. There are two main protective systems in the upper and lower respiratory tracts, i.e., physical and biological. Lung epithelial cells can be considered mostly as a physical barrier, but low molecular PM can also penetrate into interstitial spaces and induce inflammatory response (Li et al. 2010). The respiratory epithelium consists of various cell types responsible for gas exchange, surfactant synthesis, and immunological responses. Most of the air particles damage epithelial cells (Barker et al. 2014). It is established that PM-induced toxicity is strongly related to oxidative stress (Münzel and Daiber 2018). PM is capable of directly producing reactive oxygen species (ROS), causing tissue damage, inflammation, and eventually cell death (Shang et al. 2017). Experimental data show that ROS are increased in the human alveolar epithelial cell line – A549 cells, exposed to PM (Peixoto et al. 2017). Another interesting feature of PM toxicity is altered autophagy (Pesonen and Vähäkangas 2019), which is a self-digestion lysosome-related process aimed at reprocessing damaged organelles and other cellular constituents accumulated in a double-membrane vesicles called autophagosomes (Ramesh et al. 2019). In healthy cells, damaged cellular components are degraded in lysosomal pathways, but in pathology, altered autophagy may promote inflammation and further cell damage. It has been shown that autophagy can be stimulated by redox imbalance, including ROS, but also by nutrient deprivation and infections (Abounit et al. 2012; Azad et al. 2009). In recent years, evidence accumulates in support of a notion that PM-induced ROS are important to autophagy (Levonen et al. 2014; Filomeni et al. 2010), but it is still unclear of how ROS and inflammation drives autophagy in PM-exposed cells. Therefore, in this study we seek to explore the mechanisms of PM-induced ROS and autophagy using standardized urban dust (UD) and the human alveolar epithelial A549 cells.

2 Methods

2.1 Cell Culture

A549 (ATCC® CCL185™) cells grown in Dulbecco's Modified Eagle's Medium (F12K/FCS) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 were used in this study. For particular experiments, the cells were plated out onto 6- or 24-well plates and were grown in culture media supplemented with coarse carbon black (CB) or with urban dust (UD) for 24 h (both 200 $\mu g \cdot ml^{-1}$).

2.2 Cell Treatment

The conditioned media were prepared using a commercial standardized UD, purchased from the National Institute of Standards and Technology (Gaithersburg, US) and a CB, Huber 990, a primary particle diameter of 260 nm, purchased from H. Haeffner and Co. Ltd. (Chepstow, UK). The latter was used as a reference substance. According to the Certificate of Analysis of Standard Reference Material 1649b, the particle size of UD is within a range of 0.2–110 μm, with the mean particle size of about 10 μm. For experiments, particles were suspended in the cell culture medium at a concentration of 200 μg/ml and were sonicated in a Bandelin Sonopuls ultrasonic homogenizer (Berlin, Germany) for 30 s prior to use. The UD and CB-conditioned media were used within 5 min of preparation. In some experiments, A549 cells were pre-treated overnight with 100 μM buthionine sulfoximine (BSO) to deplete intracellular glutathione (GSH) levels. Cell-free controls were included to each experiment in order to assess the interference of particles with each assay.

2.3 Cell Viability and Proliferation

Cell viability was assessed with 3-[4,5-dimethylthiazolyl-2] 2,5-diphenyltetrazolium bromide (MTT) assay (Niks and Otto 1990) and by flow cytometry estimation of proliferating cell numbers. The MTT and cell cycle assays were performed after 24 h of cell treatment with UD or CB. In the MTT test, changes in absorbance due to formazone production in viable cells were measured using a double beam Perkin Elmer spectrophotometer (Waltham, MA) at 570 nm, with a 630 nm reference wavelength. Cell viability was estimated as a percentage of the control that was considered 100% viable. To quantify alterations in growth rates, cells were stained for 30 min with propidium iodide (PI; 50 μg per ml) in TRIS buffer (100 mM; pH 7.5), containing 0.1% potassium cyanide, 0.01% NP-40 detergent, 40 μg per ml Type III-A RNAse, and 0.1% NaN_3. The DNA profiles in particular cells and cell cycle analysis were performed in the aligned FACSCanto II, flow cytometer (BD Biosciences Systems; San Jose, CA), equipped with an argon laser operating at 488 nm with adjusted forward angle and side light scatter. PI fluorescence was measured in 5,000–10,000 cells, and DNA fluorescence histograms were analyzed by cell cycle software (Flowing Software v2.5; Turku, Finland). The cells were quantified by their relative distribution in the S-phase (DNA synthesis) and G2/M (post DNA synthesis/mitosis) phases of the cell cycle and were assigned as proliferating cell fractions.

2.4 Oxidative Stress

Intracellular generation of reactive oxygen intermediates was quantified in control cells and in cells treated with UD and CB using dichlorodihydrofluorescein diacetate (H_2DCFDA) (Sigma-Aldrich; St. Louis, MO) according to Ubezio and Civoli (1994). The cells were loaded with 5 μM H_2DCFDA for 30 min, washed, resuspended in phosphate-buffered saline, and assayed by flow cytometry. Green dichlorofluorescein (DCF) fluorescence was captured on FL1 channel and registered as histograms of fluorescence distribution.

2.5 Autophagy and Heat Shock Protein 70

Microtubule-associated protein 1A/1B-light chain 3 protein (LC3) and heat shock protein 70 (Hsp70) were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis – immunoblotting and by flow cytometry using appropriate rabbit monoclonal antibodies recognizing human Hsp70 (Abcam; Cambridge, UK) or LC3 proteins (Cell Signaling Inc.; Danvers, MA) and positive and negative controls. For Western blots, 10 μg (for Hsp70) or 20 μg (for LC3) of cell homogenate proteins were separated in reducing conditions by 10% or 20% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE), respectively. The proteins were transferred onto polyvinylidene difluoride membranes and incubated with specific secondary antibodies linked to alkaline phosphatase. The bound complexes were detected using BCIP (Sigma Chemical Co., Poznan, Poland) and quantified using Image Quant software (BioRad; Warsaw, Poland). A constitutively expressed protein, β-actin, served as a loading control, and the data were quantified in respect to β-actin expression. Samples were run in duplicate on each gel, and the mean values ±SD were expressed as 100 relative units to compare the data from different experiments. For flow cytometry analysis, cells were fixed in 0.01% formaldehyde for 10 min, permeabilized with 0.01% NP-40 in phosphate-buffered saline (PBS), washed with PBS, stained with specific monoclonal antibodies against LC3 (LC3A/B rabbit mAb, Alexa Fluor 488 Conjugate; Cell Signaling Inc., Danvers, MA) or with Hsp70-specific antibody bound to FITC (Abcam, Cambridge, UK), and incubated for 30 min at 4 °C. The cells were then washed, centrifuged, and resuspended in 500 μl of ice-cold

PBS containing 10% fetal calf serum (FCS) and 0.1% NaN_3. Samples were analyzed with a FACSCanto II flow cytometer (BD Biosciences Systems; San Jose, CA) equipped with a standard filter setup.

2.6 Apoptosis

A fluorescein isothiocyanate (FITC)-conjugated annexin V (Clontech Labs, Takara BioEurope; Saint-Germain-en-Laye, France) was used to detect apoptotic cells (Demchenko 2012). The A549 cells were harvested, washed twice with PBS pH 7.4, incubated in annexin V-labeling solution (final annexin V concentration, 0.5 μg/ml), washed again, and analyzed with a FACSCanto II flow cytometer (BD Biosciences Systems; San Jose, CA). Green FITC fluorescence was captured on FL1 channel through a 530 nm, 30 nm bandwidth band-pass filter. All analyses were performed at a low rate settings, with <1,000 events/s. Experimental data were plotted as fluorescence histograms and were analyzed using the Flowing Software v2.5 (Turku, Finland).

2.7 Proteins and Glutathione (GSH) Levels

Homogenate proteins were measured using the bicinchoninic acid (BCA) kit (Sigma-Aldrich; Poznan, Poland). GSH was quantified in deproteinated samples using an assay kit (Cayman Chemical; Ann Arbor, CA) that contains glutathione reductase and 5,5′-dithio-bis-2-nitrobenzoic acid and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB) measured at 405 nm.

2.8 Data Analysis

Data were expressed as means ±SD of 6–10 assays. Statistical differences were assessed using one-way or two-way analysis of variance ANOVA, followed by the Bonferroni post hoc test for selected pairs of data. A p-value < 0.05 defined statistically significant changes. Statistical analysis was performed using a commercial Statistica v6.0 package (StatSoft; Tulsa, OK).

3 Results

Table 1 and Fig. 1 show the effect of CB and UD on cell viability (MTT test), proliferation (S + G2/M cells), oxidative stress (DCF fluorescence), apoptosis (annexin binding), Hsp70 expression, and autophagy (LC3 expression) in A549 cells treated for 24 h with 200 $\mu g \cdot ml^{-1}$ CB or UD. Moreover, experiments with A549 cells pre-treated for 24 h with BSO to deplete GSH also are included. In the cells pre-treated with BSO, GSH levels decreased by about 30%, and a similar decrease was observed in UD-treated cells (results not shown). The UD, but not CB, decreased cell viability by about 27% (Fig. 1, panel a) ($p < 0.01$), and a similar effect was observed in the cells pre-treated with BSO ($p < 0.01$). Cell proliferation was affected neither by CB nor UD (Fig. 1; panel b). However, when the A549 cells were pre-treated with BSO and then treated with UD, a significant reduction by about 15% ($p < 0.05$) of S + G2/M cells was noticed. Both BSO and UD induced oxidative stress in A549 cells, but these effects were not additive when both compounds were applied sequentially to the cells (Fig. 1; panel c). Concerning the induction of apoptosis, a slight increase of annexin binding to the cell membrane was detected only in the cells pre-treated with BSO and then treated with UD (Fig. 1; panel d). The maximum number of apoptotic cells was about 9% ($p < 0.05$). BSO increased both Hsp70 (by about 33%; $p < 0.05$) and autophagy (about sixfold; $p < 0.01$), while UD induced comparable increases (by more than two-fold) in both groups. The effects of BSO and UD on autophagy, but not on Hsp70, were additive ($p < 0.01$), which was also noticed in the Western blotting, while CB partly normalized increased autophagy induced by BSO ($p < 0.01$).

Table 1 The effects of urban dust (UD) and carbon black (CB) on cell viability (MTT test), proliferation (S + G2/M cells), oxidative stress (dichlorofluorescein fluorescence), apoptosis (annexin binding), Hsp70 (flow cytometry), and autophagy (LC3 expression; flow cytometry) in human alveolar epithelial cell line A549. Naïve or buthionine sulfoximine-pre-treated cells (glutathione depleting agent; 24 h; 100 $\mu g \cdot ml^{-1}$) were grown for 24 h in medium supplemented with 200 μg/ml UD or CB. The results of MTT were quantified photometrically; all other data were quantified in flow cytometry and are shown as fractions (%) of positive or negative cells (proliferation, apoptosis). Median fluorescence intensities were also calculated (oxidative stress, Hsp70, and autophagy) and were shown as means ±SD of 3–5 experiments

	Cell viability (%)	Proliferation (%)	Oxidative stress (relative units)	Apoptosis (%)	Hsp70 (relative units)	Autophagy (relative units)
Control	100 ± 8	55 ± 7	100 ± 38	5 ± 1	100 ± 11	100 ± 45
BSO	102 ± 10	51 ± 6	130 ± 41∗	4 ± 2	133 ± 9∗	600 ± 223∗∗
CB	94 ± 7	56 ± 5	111 ± 61	6 ± 2	105 ± 13	132 ± 54
UD	73 ± 6∗∗	54 ± 5	182 ± 65∗∗	5 ± 2	222 ± 18∗∗##^^	252 ± 112∗∗##
BSO + CB	88 ± 8	56 ± 6	116 ± 63	4 ± 1	80 ± 21∗^	334 ± 126∗∗##^^
BSO + UD	8 ± 6∗∗	47 ± 4∗	173 ± 58∗∗#	9 ± 2 ∗∗# ^	266 ± 32∗∗##^^	1,122 ± 478∗∗##^^

$*p < 0.05$; $**p < 0.01$ for comparisons to the corresponding control cells;
$\#p < 0.05$; $\#\#p < 0.01$ for comparisons to the corresponding BSO-treated cells;
$\hat{}p < 0.05$; $\hat{}\hat{}\ p < 0.01$ for comparisons to CB or UD, respectively

4 Discussion

Airborne PM are now a major public health concern. Contaminated air may contain a plethora of reactive compounds including hydrocarbons, metals, and fine and ultrafine particles which, binding to cell membranes, are capable of producing ROS, alter cell maturation and growth, induce apoptosis or necrosis, affect autophagy, and produce inflammation and cancer (Ali et al. 2018; Münzel and Daiber 2018; Santibáñez-Andrade et al. 2017). Consequences of exposure to PM are highly variable, but increasing evidence supports the notion that PM-induced ROS increase and a resulting redox imbalance may have highly erosive, time-dependent effects. Oxidative stress has been detected not only in experimental models of airborne toxicity but also in clinical samples acquired from exposed individuals (Tao et al. 2003). It has been shown that ROS may affect autophagy (Levonen et al. 2014; Filomeni et al. 2010), which is relevant to the homeostatic balance of cells exposed to stress. Consequences of acute and not severe toxic stress can usually be repaired, while extended stress usually exceeds cellular adaptive capacity and causes cell death. Consequently, autophagy may be equally involved in cell protection as in cell death. The role of autophagy in toxicology has been described, and its importance in PM-induced respiratory diseases is now recognized (Ryter and Choi 2010). However, the exact mechanisms through which PM triggers biochemical and functional responses and activates chronic inflammation or cancer remain elusive.

In this study we sought to explore the mechanisms of PM-induced ROS, redox imbalance, and autophagy using standardized UD and human alveolar A549 cell line. The cells pre-treated with BSO had a lower level of GSH by about 30%. A similar decrease in GSH was noticed in the UD-treated cells, but not in the cells grown with a coarse CB which is considered a chemically neutral and nontoxic substance (Megido et al. 2016). Similar effects have been earlier described in alveolar macrophages by Geng et al. (2005) and Zhang et al. (2015), where $PM_{2.5}$ produce a dose-dependent decline of GSH and increase oxidative stress. In those studies 300 μg/ml of $PM_{2.5}$ decreased GSH by about 33%, while a lipid peroxidation product, malondialdehyde, increased by about 47%. In the present experimental model, UD decreased intracellular GSH by about 30% and diminished cell viability by about 27%. However, a similar

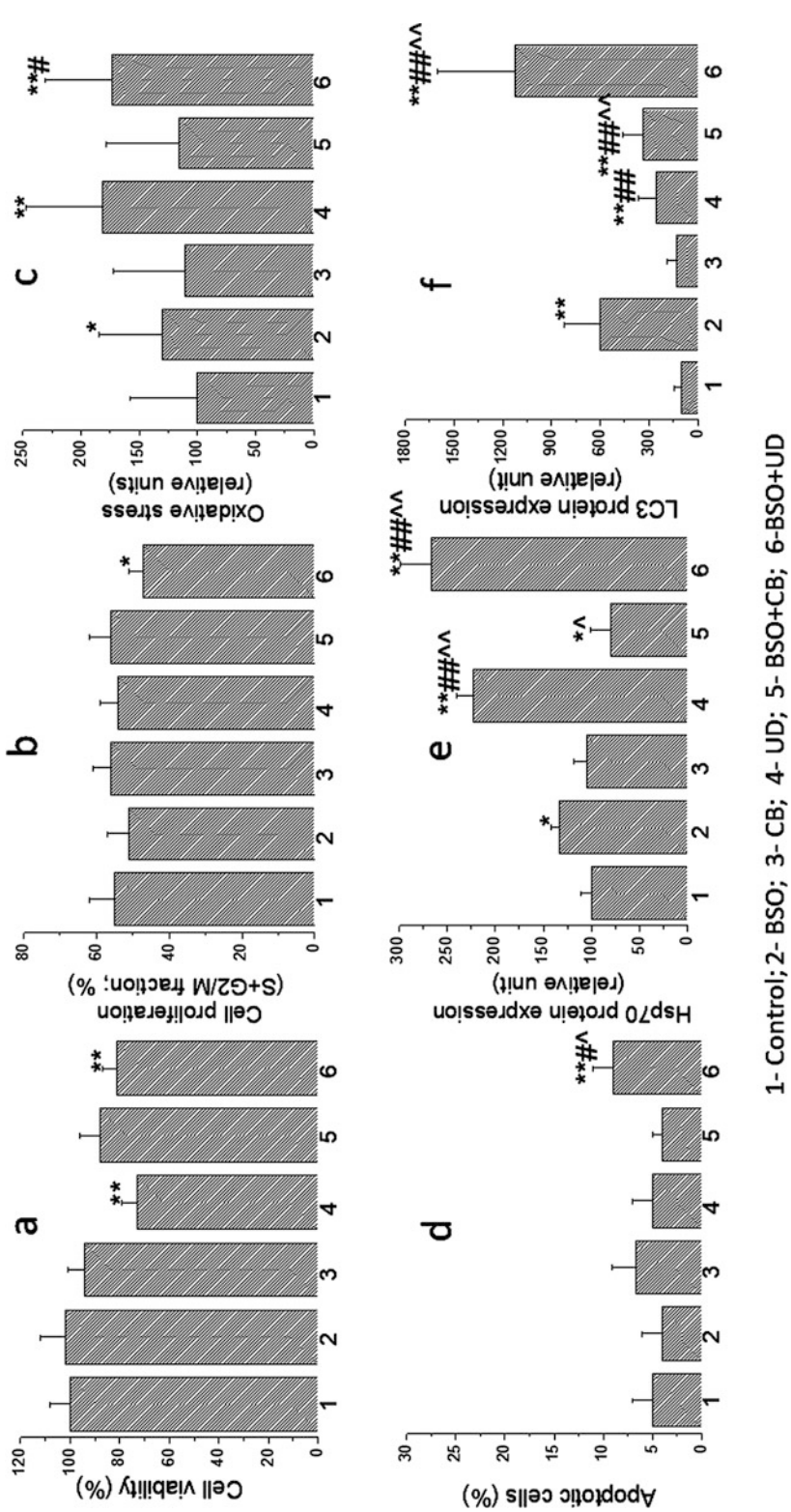

Fig. 1 The effects of urban dust (UD) and carbon black (CB) on cell viability (**a**), proliferation (**b**), oxidative stress (**c**), apoptosis (**d**), Hsp70 (**e**), and autophagy (**f**) in human alveolar epithelial cell line A549. Cells were treated for 24 h with 200 μg/ml UD or CB. In some experiments, the cells were pre-treated for 24 h with 100 μg/ml buthionine sulfoximine (BSO) to deplete their glutathione. Except for MTT test, all data were quantified in flow cytometry and are shown as fractions (%) of positive or negative cells (proliferation, apoptosis). Median fluorescence intensities were calculated (oxidative stress, Hsp70, and autophagy) and are shown as means ± SD of 3–5 experiments.

$*p < 0.05$; $**p < 0.01$ for comparisons with the corresponding control cells;
$\#p < 0.05$; $\#\#p < 0.01$ for comparisons with the corresponding BSO-treated cells;
$\wedge\wedge p < 0.01$ for comparisons with CB or UD, respectively

decrease in GSH by BSO was without a significant effect on cell survival. Moreover, when both GSH-depleting compounds, BSO and UD, were sequentially applied to the cells, UD toxicity was not enhanced. It is, therefore, possible that GSH depletion is not a prerequisite for acute UD toxicity in A549 cells. Cell proliferation was unaffected by CB and UD. However, when A549 cells were pre-treated with BSO and then treated with UD, a significant reduction in the number of dividing cells was noticed. The experiments with low molecular size PM have indicated that its low concentration suffices to produce G2/M arrest in cultured epithelial cells (Gualtieri et al. 2010). In the present study, proliferation of A549 cells was inhibited by UD only when the cells were pre-treated with BSO. In the same group of cells, a slight increase in apoptotic cell numbers was found, but the maximum fraction of apoptotic cells was less than 10%, and it seems that the unspecific annexin binding cannot be excluded in that type of toxic stress. It is known that PM is able to induce oxidative stress (Münzel and Daiber 2018).

In the present study, pro-oxidative alterations were detected in cells treated with BSO, in cells grown with UD, and in cells treated successively with both compounds. However, there was no additive effect of BSO and UD on oxidative stress. An early marker of oxidative stress and unfolded protein response, Hsp70 protein, also increased in the groups of A549 cells with elevated ROS, which may indicate oxidative protein damage. The important role of oxidative stress in cellular responses is evidenced in the experiments with cell exposure to airborne pollutants and antioxidants which partly blocked PM-induced DNA damage and G2 arrest (Longhin et al. 2013). In the present study, BSO produced very high increase in LC3 expression, which can be indicative of increased autophagy. CB was without an effect on LC3, while UD stimulated autophagy by more than twofold. It is possible that enhanced autophagy may represent an adaptive reaction to stress. Recently, enhanced autophagic activity has been linked to reparation of damaged DNA. However, excessive activation of autophagy due to hypoxia-ischemia or starvation has also been observed in cells undergoing cell death (Albrecht et al. 2019). We have previously shown that UD may induce DNA damage in A549 cells, including both single- and double-strand breaks that occurred as early as 1 h of cell exposure to UD and persisted for many hours in the presence of UD (Mroz et al. 2008). It is possible that cell exposure to UD may generate, to some extent, a genotoxic insult, followed by increased autophagy and subsequent protein translocation from the cytosol to the nucleus to repair and maintain genome integrity.

In summary, this study provides evidence for the role of redox imbalance in urban dust cytotoxicity and in autophagy in epithelial cells. Nevertheless, further studies are needed to clarify the relationship between redox-dependent and redox-independent aspects of urban dust toxicity and their relation to autophagy, which seems essential for the understanding of chronic inflammation and genomic stability in lung epithelial cells exposed to environmental pollutants.

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

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors. The study has been approved by an institutional research ethics committee.

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Autologous Platelet-Rich Plasma Reduces Healing Time of Chronic Venous Leg Ulcers: A Prospective Observational Study

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Abstract

The study investigated whether the application of dressings with autologous platelet-rich plasma (PRP) would reduce the healing time in patients with chronic venous leg ulcers. This is a prospective observational study that included 100 patients diagnosed with lower extremity venous insufficiency complicated by ulceration of a leg or foot, who had been after angioplasty of stenotic artery. Patients were divided into two groups of 50 each: treated with PRP (study group) and treated with conventional hydrocolloid dressings (control group). We followed the wound changes at Day 10, Day 20, and Day 30 of treatment and compared them with the baseline appearance at Day 0. We evaluated the appearance, area, and depths of wounds with ultrasound. The granulation process was examined histologically to document skin formation and wound tissue neovascularization. The findings were that treatment with PRP dressings resulted in a significant progressive reduction in ulcer size, irrespective of the ulcer's initial size, compared to treatment with conventional dressings. Further, the best effect of PRP was noticed in the category of largest wounds. After a month of treatment with PRP dressings, more than 50% of all ulcers were completely healed. The young epidermis appeared together with the granulation tissue, and the formation of dermis took shape after 20 days of treatment. We conclude that the use of PRP dressings is a safe, nonsurgical adjunctive procedure for treating chronic

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venous leg ulcers. The potential benefit of PRP dressings over conventional ulcer treatment requires further in-depth exploration.

Keywords
Chronic venous insufficiency · Granulation tissue · Healing time · Inflammation · Leg ulcers · Platelet-rich plasma · Skin formation · Wound management

1 Introduction

Chronic venous insufficiency is a pathologic process of the venous system in lower extremities characterized by incompetent venous valves and venous hypertension leading to edema, trophic skin changes, and venous ulcers. The prevalence of chronic venous insufficiency in the industrialized countries is enormously high, ranging from 20% to 64% (Wittens et al. 2015; Criqui et al. 2003). Venous leg ulcers are the most severe manifestation of venous insufficiency. The ulcers are slow to heal and have a high recurrence rate. Consequently, they pose a significant physical, emotional, and socioeconomic burden for patients, families, and the healthcare system. In tandem with a worldwide increase in longevity and obesity, the incidence of venous leg ulcers is growing (van Rij et al. 2008; Margolis et al. 2002). About 2.5–3.0 million Americans are affected by venous insufficiency and at least 600,000 suffer from chronic leg ulcers, resulting in an annual economic burden of up to $15 billion (Rice et al. 2014; Korn et al. 2002).

Numerous treatment modalities have been proposed for treating venous leg ulcers. Conventional methods include a topical treatment such as debridement, dressing, compression therapy, antimicrobials, and antiseptics. Systemic therapy includes the administration of antibiotics, pharmacological agents, and skin grafting. Alternative therapies are also used such as electromagnetic fields, hyperbaric oxygen therapy, intermittent pneumatic compression, lasers and infra-red light, negative pressure therapy, and venous leg surgery. None of these treatments are considered entirely satisfactory, and there are insufficient data to draw final recommendations for treatment of chronic venous leg ulcers (Athanerey et al. 2017).

Studies on the molecular mechanisms involved in tissue regeneration have led to the development of the new therapeutic methods for venous leg ulcers. Specifically, growth factors contained in platelet granules are conducive to tissue repair in chronic wounds because they act by regulating cellular proliferation, migration, and differentiation in addition to the synthesis of the extracellular matrix. Recent studies have revealed that growth factors modulate the healing of both chronic and acute wounds by interaction with cellular tissue receptors (Martinez et al. 2015).

The application of autologous platelet-rich plasma (PRP), which contains a greater concentration of growth factors than whole blood, has gained popularity in plastic and orthopedic surgery since it is effective and safe in terms of better survival of fatty tissue and bone grafts (Hsu et al. 2013; Sommeling et al. 2013). PRP contain a high concentration of growth factors, which are released from platelets, that contribute to the tissue regeneration process (Martinez-Zapata et al. 2016). The ability of PRP to induce migration of specific cell types could be harnessed to stimulate the healing process of surgical wounds and skin ulcerations (Roubelakis et al. 2014). Yet data on the efficacy of PRP in treatment of chronic venous leg ulcers are scarce. Thus, this study seeks to determine whether application of autologous PRP would reduce healing time in patients with such ulcers.

2 Methods

2.1 Study Design

The prospective observational study included 100 patients diagnosed with lower extremity venous insufficiency complicated by ulceration

of a leg or foot, who had been after angioplasty of stenotic artery. The starting time of ulcers development was not considered an essential factor since a vast majority of ulcers were recurrent. Patients were subdivided into two groups: active treatment with PRP and a control group treated with a conventional method using hydrocolloid dressings (AQUACEL Ag Surgical dressing; ConvaTec Inc., Greensboro, NC); 50 patients each. The main inclusion criteria were ulcer size not exceeding 5 cm^2 lower limb ischemia clinically and radiologically evidenced with CT angiography, recent successful revascularization, and blood creatinine <1.0 mg/dL. Patients with ischemic ulcers were excluded.

We evaluated the appearance, size, and the depths of ulcer wounds with ultrasound. The granulation process was examined histologically. Duration of ulcers ranged from 6 to 16 months, with an average of 7.3 months. The area of ulceration was calculated by tracing its outline the ulcer size on carbon paper and then transferring it onto scaled grid paper. The size of a dressing fortified with PRP available in the healing kit (Regeneris Medical; North Attleboro, MA) determined the maximum size of the wound treated. Dry necrosis of the wound was present in both groups. All patients underwent a surgical debridement of the wound to remove nonviable tissue in order to decrease the bacterial load and stimulate epithelialization.

2.2 Study Procedures

PRP was obtained by drawing 18 mL of the patient's blood from a venipuncture. The blood was centrifuged to separate erythrocytes and leukocytes from plasma containing platelets. We added a branded reagent to the plasma, which activated the platelets to produce growth factors, applied PRP gel onto the ulcer wound cleaned from necrotic debris, and washed with physiological saline. The wound was covered with a hydrocolloid AQUACEL dressing, which was maintained for 10 days and then replaced with a fresh one. We repeated the whole procedure after 20 and 30 days of treatment. Patients were treated in like manner, except that the PRP-fortified dressing was omitted.

2.3 Data Collection and Analysis

The time to heal was taken as a primary outcome. We performed a power analysis to calculate the sample size with regard to the primary outcome, assuming α error of 5% and β error of 20%. The calculation indicated that 45 lesions in each group were necessary for the assessment. Another outcomes consisted of changes in size and depth of wounds, and the formation of granulation tissue, examined histologically, and ulcer epithelialization, examined with a 50 MHz ultrasound probe. In addition, we examined the formation of skin layer and wound vessels. Ulcers were stratified into four increasing categories depending on the initial area size, ranging from 1 to 5 cm^2. The outcomes were assessed at four different time points: baseline, and after 10, 20, and 30 days of treatment.

Continuous data were expressed as means ±SD and categorical data as numbers or percentages. Univariate analysis was performed using Student's *t*-test for continuous variables and Chi-square tests for categorical variables. One-way ANOVA was used for multiple comparisons. Multivariate analysis using linear and logistic regressions also were performed as required. A p-value <0.05 defined statistically significant changes. A commercial statistical SPSS v21 package was used for the analyses (IBM; Armonk, NY).

3 Results

We treated and analyzed a total of 100 venous ulcers in lower extremities in 100 patients. The PRP and control groups were grossly matched concerning age and gender (Table 1) as well as categories of ulcer wound size (Table 2).

Table 1 Demographic data of patients with leg ulcers due to chronic venous insufficiency treated with platelet-rich plasma (PRP) dressings and with conventional hydrocolloid dressings (control group)

	PRP group ($n = 50$)	Control group ($n = 50$)
Gender (F/M) (n)	16/34	11/39
Age range F/M (year)	55–86/53–89	57–79/54–79
Cigarette smokers (n)	19	17
Arterial hypertension (n)	26	21
Coronary heart disease (n)	5	7
History of myocardial infarction (n)	2	1
Atherosclerosis of lower extremities (n)	24	39
Renal failure (n)	15	17
Age of diabetes mellitus onset (year)	7–21	9–18
Insulin therapy (n)	47	49
Oral hypoglycemic drugs (n)	3	1

The PRP-treated group demonstrated a significantly better healing on Day 10 of treatment in the categories of the smallest and largest wounds compared to the control group ($p < 0.01$). The wound area was significantly smaller in the PRP-treated group on Day 20 and Day 30, compared to the control group, in all size categories ($p < 0.01$ for all). We found the best effect on wound healing of PRP-fortified dressing in the largest wound size category (Table 2). The area of ulcers appreciably decreased in all individual patients already at Day 10 of treatment with PRP dressings, the decrease became greater at Day 20, and the ulcers were completely healed in 28 (56%) out the 50 patients at Day 30 (Table 3).

The initial phase of healing, i.e., granulation, was observed as early as 10 days after PRP application (Fig. 1a). It involved the ingrowth of granulation tissue and a reduction in wound exudate. We also noticed less fibrin at the bottom of a wound and reduced swelling of the wound edges. The formation of dermis accelerated in the second phase of healing after 20 days, along with enhance neovascularization (Fig. 1b). The third phase of healing, observed after 30 days' treatment, consisted of the formation of epidermis over a layer of the freshly formed dermis (Fig. 1c). Granulation tissue contained a rich microvasculature (Fig. 2a), with clearly enhanced blood supply to tissue (Fig. 2b). There were no observable complications during the study period. Occasional minor injuries to the wound did not delay healing. Eventually, all the ulcer wounds healed, and the average duration of therapy was 35 days. No additional wound treatment was required.

4 Discussion

In this study, patients with venous leg ulcers who received treatment with the addition of PRP exhibited a significantly shorter time to healing. Overall, we achieved excellent results by applying a standardized PRP protocol for treatment of chronic venous leg ulcers, which had been unresponsive to conventional treatment modalities. All the PRP-targeted patients had a complete wound healing within 5 weeks, with an improvement in all healing parameters. The procedure was safe as it did not result in any deterioration in wound status. Venous ulcers are a severe complication of chronic venous insufficiency. They are characterized by chronic inflammation and are resistant to local therapies (O'Meara et al. 2000). An appropriate surgical procedure to reduce venous hypertension at the ulcer site and local debridement to remove necrotic tissues are essential for treatment success (Smith 2006). A better understanding of the pathophysiology of chronic venous leg ulcers healing should help define the therapy of choice and the optimal care planning.

A role of various growth factors in wound healing has long since been acknowledged. However, growth factors, one by one, have been shown to fail to respond to multiple needs of

Table 2 Categories of wound area of leg ulcers due to chronic venous insufficiency in patients treated with platelet-rich plasma (PRP) dressings and in the control group treated with conventional hydrocolloid dressings at successive time points of healing

Measurement time point	Group	Category of wound area (cm^2)	Mean area ± SD (cm^2)	*n*
Baseline	PRP	1.0–2.0	1.7 ± 0.2	6
		2.1–3.0	2.5 ± 0.4	6
		3.1–4.0	3.7 ± 0.3	13
		4.1–5.0	4.5 ± 0.3	25
		Overall	3.7 ± 1.0	50
	Control	1.0–2.0	1.9 ± 0.2	7
		2.1–3.0	2.8 ± 0.1	5
		3.1–4.0	3.5 ± 0.3	15
		4.1–5.0	4.7 ± 0.3	23
		Overall	3.7 ± 1.1	50
Day 10	PRP	1.0–2.0	1.0 ± 0.2	6
		2.1–3.0	1.7 ± 0.4	6
		3.1–4.0	2.7 ± 0.3	13
		4.1–5.0	3.4 ± 0.4	25
		Overall	2.7 ± 1.0	50
	Control	1.0–2.0	1.3 ± 0.2	7
		2.1–3.0	2.0 ± 0.2	5
		3.1–4.0	2.5 ± 0.3	15
		4.1–5.0	3.8 ± 0.3	23
		Overall	2,9 ± 1.0	50
Day 20	PRP	1.0–2.0	0.5 ± 0.3	6
		2.1–3.0	1.0 ± 0.4	6
		3.1–4.0	1.7 ± 0.5	13
		4.1–5.0	1.8 ± 0.3	25
		Overall	1.5 ± 0.6	50
	Control	1.0–2.0	0.9 ± 0.2	7
		2.1–3.0	1.7 ± 0.1	5
		3.1–4.0	1.9 ± 0.2	15
		4.1–5.0	2.9 ± 0.2	23
		Overall	2.2 ± 0.8	50
Day 30	PRP	1.0–2.0	0.1 ± 0.2	6
		2.1–3.0	0.3 ± 0.4	6
		3.1–4.0	0.9 ± 0.4	13
		4.1–5.0	1.1 ± 0.2	25
		Overall	0.8 ± 0.4	50
	Control	1.0–2.0	0.8 ± 0.2	7
		2.1–3.0	1.3 ± 0.3	5
		3.1–4.0	1.7 ± 0.2	15
		4.1–5.0	2.1 ± 0.2	23
		Overall	1.7 ± 0.5	50

nonhealing tissues. For that reason, interventions such as PRP therapies are currently under investigation in order to target a broad set of factors involved in the different stages of healing, which are often interrupted by a range of comorbidities. PRP carries molecules that play a crucial role in many wound healing phases such as hemostasis, inflammation, cell migration and proliferation, extracellular matrix production, and tissue remodeling (Steed 1995).

Table 3 Changes off baseline (Day 0) in the wound area of leg ulcers due to chronic venous insufficiency at successive time points of healing in individual patients treated with platelet-rich plasma (PRP) dressings

	Day 0	Day 10	Day 20	Day 30
Patient	Wound area (cm^2)			
1	3.6	2.8	1.7	0.6
2	2.8	2.1	1.2	Healed
3	3.9	3.0	2.3	0.4
4	2.3	2.0	1.4	Healed
5	4.8	4.0	3.3	1.1
6	3.3	2.6	1.9	Healed
7	1.9	1.3	0.5	Healed
8	3.7	3.0	1.2	Healed
9	4.0	3.5	2.7	0.6
10	3.8	3.5	2.4	1.0
11	4.1	3.1	2.0	0.3
12	2.5	1.8	0.8	Healed
13	3.1	2.6	1.2	0.2
14	1.9	0.8	0.1	Healed
15	2.6	1.6	0.5	Healed
16	4.8	3.9	2.1	1.2
17	4.2	3.1	2.0	0.8
18	1.5	0.8	0.2	Healed
19	2.2	1.0	0.4	Healed
20	3.1	2.6	1.6	0.3
21	1.9	1.0	0.4	Healed
22	2.6	1.4	0.5	Healed
23	4.5	3.6	2.2	1.0
24	3.9	2.8	1.0	0.3
25	2.7	1.4	0.5	Healed
26	3.0	2.1	1.0	0.3
27	2.4	1.3	0.5	Healed
28	2.6	1.6	0.5	Healed
29	1.5	0.6	0.2	Healed
30	5.0	3.9	2.3	1.1
31	2.3	1.3	0.3	Healed
32	2.6	1.4	0.6	Healed
33	2.1	1.3	0.4	Healed
34	2.8	1.5	0.6	Healed
35	2.0	1.3	0.4	Healed
36	4.6	3.6	2.7	1.2
37	3.5	2.3	1.1	0.3
38	2.1	1.3	0.5	Healed
39	1.8	0.9	0.2	Healed
40	2.3	1.0	0.3	Healed
41	2.0	0.9	0.4	Healed
42	4.5	3.6	2.0	1.0
43	3.3	2.1	1.0	0.2
44	1.7	1.0	0.2	Healed
45	4.9	3.8	2.1	1.1
46	2.3	1.1	0.2	Healed
47	3.0	1.9	1.0	0.4

(continued)

Table 3 (continued)

Patient	Day 0	Day 10	Day 20	Day 30
	Wound area (cm^2)			
48	2.2	1.0	0.3	Healed
49	4.6	3.8	2.3	1.1
50	3.2	2.4	1.3	0.5

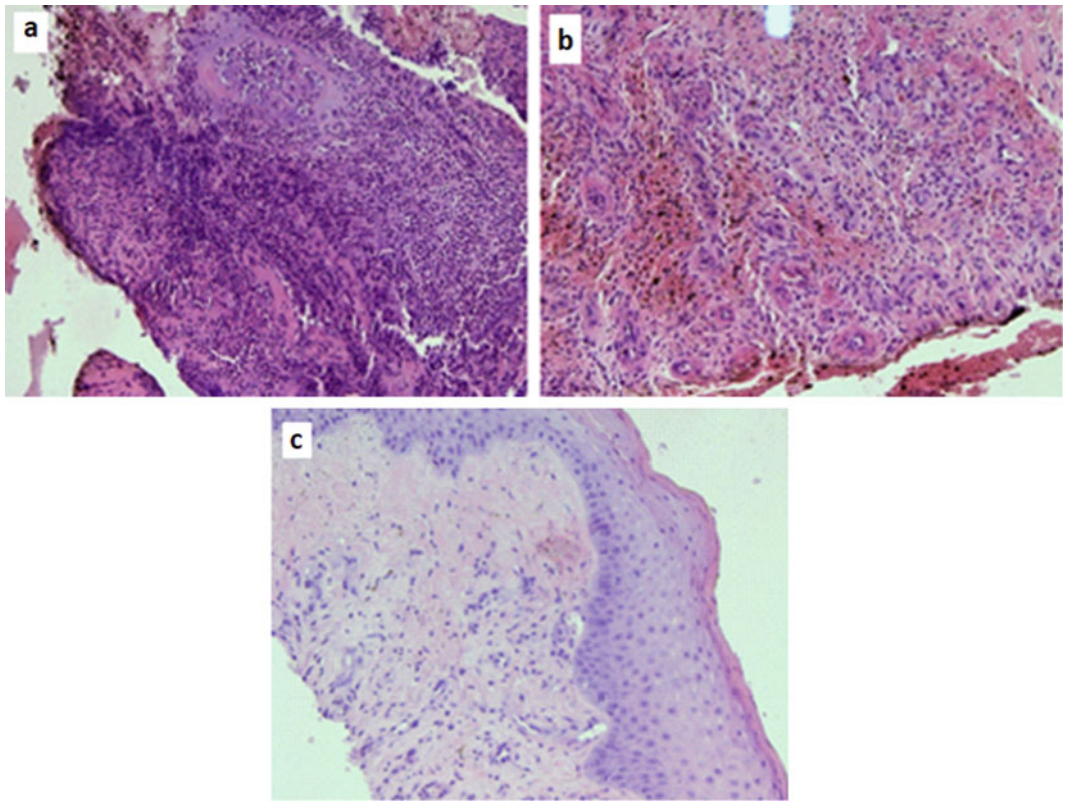

Fig. 1 Sequential phases of healing of a leg ulcer in a patient with chronic venous insufficiency after application of autologous platelet-rich plasma (PRP) dressings: (**a**) initial phase of healing – granulation tissue at Day 10; (**b**) continuing formation of dermis in well-perfused granulation tissue at Day 20; (**c**) right-formed layers of dermis and epidermis at Day 30

The results reported herein are consistent with several other recently published studies. Babaei et al. (2017) have noticed the formation of healthy granulation tissue and early complete closure of every wound in 150 patients with diabetic foot ulcers after topical application of PRP. Likewise, Suthar et al. (2017) have noticed that treatment of nonhealing ulcers of different etiologies with subcutaneous autologous PRP injections, combined with topical application of PRP gel, leads to a significant reduction in wound size as a result of suppression of cytokine release. In addition, there is a reduction in pain and inflammation at the injury site. Comparable results have been obtained after topical use of autologous PRP in secondary wounds due to necrotizing soft tissue infections (Hersant et al. 2017). Cieslik-Bielecka et al. (2018) have shown that PRP eradicates microorganisms from, and induces neovascularization in, chronic ulcers of the crural region

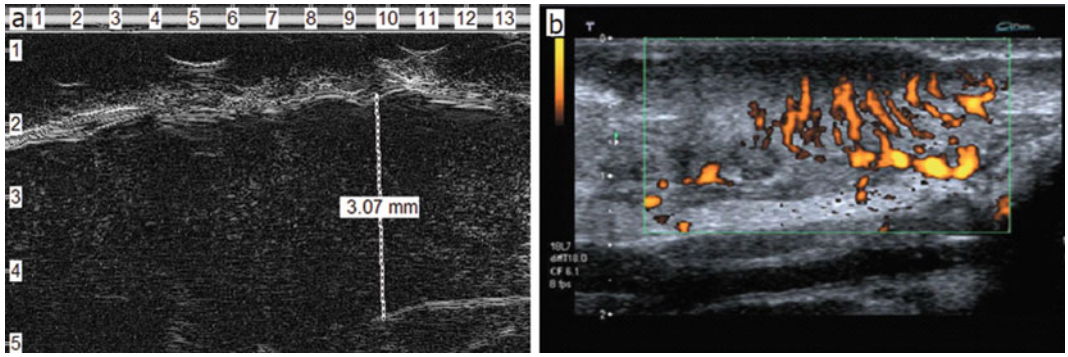

Fig. 2 Day 30 of healing of a leg ulcer in a patient with chronic venous insufficiency: (**a**) right-formed layer of dermis, indicated by a vertical line in mm; (**b**) neovascularization – increased blood flow in the granulation tissue

in AIDS patients. In another study, Man et al. (2001) have shown a quantitative improvement in human skin wound healing after topically treating cutaneous flaps with autologous PRP.

In this study, we adopted the TIME strategy for local wound treatment. The strategy consists of removal of necrotic tissues (T – tissue debridement), control of wound infection and inflammation (I – inflammatory and infection control), maintaining the wound moisturized (M – moisture balance), and wound stimulation (E – stimulation of epidermalization) (Leaper et al. 2012). The TIME strategy produces good results in recently formed wounds. Chronic wounds of more than 2 months in duration require, in addition, the use of growth factors. When using these factors, we observed not only progress in healing but also changes in the characteristics of ulcers. Wound secretion became intense and light, resembling plasma. The ulcer floor, initially flat and covered with necrotic tissues and fibrin, became rough and filled with patches of granulation tissue. Swelling and inflammation decreased significantly.

The use of autologous PRP as a biological dressing significantly increased the wound healing rate and resulted in a complete healing of chronic leg ulcers in all patients suffering from venous insufficiency. The first phase of healing consisting of granulation appeared as fast as 10 days after PRP application, along with a reduction in exudate and a layer of fibrin at the wound floor, and in swelling of wound edges. These changes might likely be due to the action of antibacterial and chemotactic cytokines whose content increases in the wound as reported by Rosner et al. (2001). Prostaglandins formed and released from platelets, potent vasodilators, may accelerate the formation of renewed layers of dermis; the process would be facilitated by a dense network of newly formed microvessels, supplying the granulation tissue with blood as depicted in Fig. 2b. We found in this study that ulcers caused by venous insufficiency in the leg of all area size healed faster when PRP-fortified dressings were applied to the wound. Further, the best effect of PRP was noticed in the largest category of wound. The findings of this study were that with the use of autologous PRP, wounds up to 3.7 cm^2 healed entirely within a month, and more massive wounds healed within 40 days of treatment onset.

A limitation of this study was a relatively short follow-up period, which makes it impossible to draw firm conclusions as to the long-term results of ulcer wound healing with the aid of autologous platelet-rich plasma. Nonetheless, we believe we have shown that platelet-rich plasma is highly effective in shortening and improving the healing process. The autologous platelet-rich plasma emerges as a safe, nonsurgical adjunct procedure for treating chronic venous leg ulcers. Additional studies performed in larger groups of patients and with extended follow-up periods are required to confirm these findings.

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

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was reviewed and approved by the Bioethics Committee of the Medical University of Warsaw in Poland (permit AKBE/127/15).

Informed Consent Written informed consent was obtained from all individual participants included in the study.

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