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ORIGINAL ARTICLE

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The clinical significance of serum oxidative stress biomarkers in breast cancer females

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ABSTRACT

Background: This study was conducted to evaluate the role of some serum oxidative stress biomarkers for breast cancer diagnosis, incidence and monitoring the effects of surgery and chemotherapy.

Methods: A total of 35 breast cancer patients (before surgery, after two weeks of surgery and after 6 cycles of chemotherapy) and 35 normal healthy controls were analyzed for serum oxidative stress markers including total antioxidant capacity (TAC), malondialdehyde (MDA), total, reduced (GSH) and oxidized (GSSG) glutathione and glutathione redox status (GSH/GSSG).

Results: The serum levels of MDA and GSSG were significantly higher in breast cancer patients than controls. The serum levels of GSH, TAC and GSH/GSSG ratio were significantly lower in breast cancer patients than controls. After surgery, the serum levels of MDA and GSSG were significantly decreased, while the serum levels of GSH were significantly increased, compared with their levels before surgery. Six cycles of chemotherapy showed the non-significant effect on the serum levels of the assayed biomarkers. ROC curve analysis demonstrated that MDA and GSH were superior to the GSH/GSSG ratio, TAC and GSSG. Increased levels of MDA and GSSG and reduced levels of GSH, TAC and GSH/GSSG ratio were found to significantly increase the risk of breast cancer.

Conclusions: All of the assayed biomarkers can be used for prediction of breast cancer with MDA and GSH being superior to the others. MDA, GSH and GSSG were able to monitor the effect of surgery. All of the assayed biomarkers were found to be associated with breast cancer risk. None of the assayed biomarkers was able to predict the effect of chemotherapy.

Key words: Breast cancer, MDA, TAC, GSH, GSSG, Glutathione redox status

Introduction

Reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, and hydrogen peroxide are metabolic by-products leaking from the complexes I and III of the mitochondrial respiratory chain [1]. Oxidative stress is considered to be involved in the pathophysiology of all cancers including breast cancer [2, 3].

Breast cancer cells are subjected to a high level of oxidative stress, both intracellular and extracellular. To ensure survival, cancer cells must acquire special adaptive mechanisms that counteract the toxic effects of free radicals exposure. These mechanisms may involve the activation of redox-sensitive transcription factors and the increased expression of antioxidant enzymes and antiapoptotic proteins. Moreover, it was revealed that different breast cancer cell types show different intracellular antioxidant capacities that may determine their ability to resist radiotherapy and chemotherapy [4].

The involvement of oxidative stress in breast carcinogenesis has not been extensively documented [5]. Panis et al. [6] reported that although a growing number of studies have focused on the relationship between breast cancer and oxidative stress, none of the previous reports determined the impact of the systemic oxidative stress status on patients with primary breast tumours, as well as if its removal could change the oxidative profiling of plasma.

One of the possible clinical applications of oxidative stress status in cancer is the use of oxidative stress markers as tumour markers [7]. The current study examined the extent of lipid peroxidation, total antioxidant capacity and the status of glutathione in serum of breast cancer patients to investigate the role of these biomarkers in the etiology, prediction, risk and monitoring the

effects of surgery and chemotherapy on breast cancer patients in an attempt to improve the clinical outcome of those patients.

Subjects and methods

Seventy females were enrolled in this case-control study. Females were divided into two groups. **Group I (breast cancer patients group)** which included 35 female patients with breast invasive ductal carcinoma of clinical stages II and III [8] (recently detected, not underwent surgery or receiving chemotherapy). Their mean age was 43.73 ± 12.2 years. Patients were recruited from the Department of Experimental and Clinical Surgery of the Medical Research Institute, Alexandria University, Egypt in the period from January 2018 till June 2018. **Group II (normal healthy control group):** It included 35 apparently normal healthy female volunteers of comparable age (42.18 ± 11.05), menstrual cycle and socioeconomic status as patients.

After having approval from the ethics committee, Medical Research Institute, Alexandria University, Egypt, signed informed consents were obtained from all subjects who agreed to participate in this study. Each patient underwent full history recording, thorough clinical examination, routine laboratory investigations including complete blood count (CBC), mammography of breast and ultrasonography of abdomen and liver, radiological investigations including X-ray chest, CT scan and bone scan when needed and fine needle aspiration cytology (FNAC) of breast mass to establish the pathological diagnosis in the patients.

The clinicopathologic data were obtained from patients' pathology reports. The collected data included tumour size, tumour pathological grade, axillary lymph node involvement, vascular invasion and status of estrogen receptor (ER) and progesterone receptor (PR). Each breast cancer patient's clinical stage was determined by the oncologist according to the tumour-nodes-metastasis (TNM) classification system [8].

All 35 breast cancer patients were subjected to modified radical mastectomy (MRM) surgery [9]. The patients received adjuvant combination chemotherapy [5-Fluorouracil, Adriamycin and Cyclophosphamide (FAC)] [10] for 6 cycles. After 6 cycles of chemotherapy, breast cancer patients were re-evaluated clinically, laboratory and radiologically to evaluate the clinical response.

Laboratory Investigations

Five-milliliter blood sample was collected once from normal healthy female volunteers and from breast cancer patients before surgery, after 2 weeks of surgery and after 6 cycles of chemotherapy. Immediately after withdrawing, blood samples were allowed to coagulate and centrifuged for 20 minutes at 3500 rpm. The separated serum samples were aliquoted, frozen at -80 °C, and stored until assayed. After thawing, each serum aliquot was assayed only once. Determination of serum levels of MDA, total antioxidant capacity, total and reduced forms of glutathione were carried out at Radiation Sciences Department, Medical Research Institute, Alexandria University, Egypt. While the serum levels of oxidized glutathione and glutathione redox status were estimated mathematically.

Determination of serum malondialdehyde concentration

Malondialdehyde (MDA) is the end product of lipid peroxidation. MDA was assayed using a ready-for-use colourimetric kit (Biodiagnostic, Egypt). MDA was quantified by its reaction with the exogenously added thiobarbituric acid (TBA) that reacts with MDA in acidic medium at a temperature of 95°C for 30 min to form thiobarbituric acid (TBA) reactive product. The absorbance of the resultant pink product was measured at 534 nm. The absorbance of each serum sample was read against a blank (which contained only TBA) and a standard (which contained MDA of conc. 10 nmole/ml). In each serum sample, the concentration of MDA was calculated according to the equation:

MDA (nmole/ml) = (Abs of serum/ /Abs of standard)*10

Determination of serum total antioxidant capacity

The degree of oxidative stress was assessed by determining total antioxidant activity using a ready-for-use colourimetric kit (Biodiagnostic, Egypt). The principle of the method depends on the reaction of antioxidants in the sample with a defined amount of exogenously added H_2O_2. The antioxidants in the sample eliminate a certain amount of the added hydrogen peroxide. The residual H_2O_2 is determined colourimetrically by an enzymatic reaction involving the conversion of the exogenously added 3, 5, dichloro-2-hydroxyl benzenesulfonate to a pink coloured product. The absorbance of each sample was read at 505 nm against a blank in which the sample was replaced with distilled water. In each sample, the total antioxidant concentration was calculated according to the equation:

Total antioxidant concentration (mM/L) = (Abs of blank-Abs of sample)*3.33

Determination of serum levels of total glutathione

The enzymatic method described by Griffith [11] was used to measure the levels of total glutathione. This is a sensitive and specific enzymatic method which depends on the oxidation of reduced glutathione (GSH)

by 5,5-dithiobis-2-nitrobenzoic acid (DTNB) to yield oxidized glutathione (GSSG) and 5-thio-2-nitrobenzoic acid (TNB). Oxidized GSSG is reduced enzymatically by the action of glutathione reductase and NADPH to regenerate GSH which reacts again. The rate of TNB formation is monitored at 340 nm and is proportional to the sum of GSH and GSSG present in the sample.

Total glutathione (U/L) = 4019 x Δ 340 nm/min

Determination of serum levels of reduced glutathione (GSH)

The level of reduced glutathione in serum was assessed using a ready-for-use colourimetric kit (Biodiagnostic, Egypt). The method is based on the reduction of 5,5` dithiobis -2-nitrobenzoic acid (DTNB) by glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm.

Serum level of GSH(mg/dl) = (A"sample")*66.66

Estimation of serum levels of oxidized glutathione (GSSG):

Oxidized glutathione (GSSG) was obtained by subtracting the values of reduced glutathione (GSH) from the values of total glutathione (tGSH) [12].

GSSG = tGSH — GSH

Estimation of serum glutathione redox status (GSH/GSSG)

The most widely used indicator of the glutathione redox status (GSH/GSSG) of the cells is according to Kirlin et al. [13]:

Redox Status = (Serum level of GSH)/ /(Serum level of GSSG)

Where [GSH] and [GSSG] are molar concentration of reduced and oxidized glutathione; respectively.

Statistical Analysis

The statistical analyses were carried out using the Predictive Analytics software (PASW statistics 18). The Non-parametric Mann-Whitney U-test was used for studying differences between breast cancer patients group and control group regarding serum levels of total antioxidant capacity (TAC), malondialdehyde (MDA), total, reduced (GSH) and oxidized (GSSG) glutathione and glutathione redox status (GSH/GSSG). The diagnostic values of assayed parameters were compared using the Receiver Operating Characteristic (ROC) curve analysis. The cut-off point for each biomarker was determined according to the best discrimination between patients and controls regarding optimal values of sensitivity and specificity using the ROC curve. Spearman correlation was carried out to explore the possible correlation between different biomarkers and clinicopathological data. Odd's ratio with a 95% confidence interval (95% CI) was calculated using the same program. P-values < 0.05 were accepted as significant.

Results

Serum levels of oxidative stress biomarkers in the controls and breast cancer patients before surgery, after two weeks of surgery and after 6 cycles of chemotherapy.

Table 1 showed oxidative stress biomarkers in serum of normal healthy control females and breast cancer females before surgery, after two weeks of surgery and after six cycles of chemotherapy. Serum levels of MDA and GSSG were significantly elevated in breast cancer patients group before surgery compared with

Table 1. Serum levels (Mean ± SE) of oxidative stress biomarkers in the controls and breast cancer patients before surgery, after 2weeks of surgery and after 6 cycles of chemotherapy

Serum biomarkers	Control group (n = 35)	Breast cancer patients group (n = 35)		
		BS	2 W	6 C
MDA (nmole/ml)	11.10 ± 0.44	20.01 ± 0.37[a]	12.27 ± 0.29[b]	13.56 ± 0.33
TAC (mM/l)	1.26 ± 0.49	0.78 ± 0.46 [a]	0.90 ± 0.40 [a]	0.83 ± 0.38 [a]
GSSG(mg/dl)	9.23 ± 0.21	10.39 ± 2.22 [a]	9.64 ± 2.91 [b]	10.29 ± 2.98
GSH (mg/dl)	21.71 ± 1.07	15.85 ± 1.08 [a]	17.14 ± 2.13 [a,b]	13.61 ± 4.01 [a]
Glutathione Redox status (GSH/GSSG)	2.38 ± 0.47	1.84 ± 0.14 [a]	1.57 ± 0.16 [a]	1.54 ± 0.13 [a]

N — sample size; BS — before surgery; 2W — after two weeks of surgery; 6C — after six cycles of chemotherapy; a — significance compared with control group; b — significance compared with breast cancer group before surgery; c — significance compared with breast cancer group after two weeks of surgery; significance was considered at p-value < 0.05.

the control group. The serum levels of MDA and GSSG were significantly decreased in breast cancer patients group after two weeks of surgery compared with their levels before surgery. A non-significant difference was found in serum levels of MDA and GSSG after 6 cycles of chemotherapy compared with their levels after two weeks of surgery.

Table 1 also showed that serum levels of TAC, GSH and glutathione redox status (GSH/GSSG) were significantly decreased in breast cancer females before surgery compared with the control group. The serum levels of reduced glutathione in breast cancer patients group after two weeks of surgery were significantly elevated compared with their levels before surgery. After six cycles of chemotherapy, none of these biomarkers significantly differed from its level after two weeks of surgery.

Comparison between serum levels of oxidative stress biomarkers as diagnostic markers in breast cancer patients using the Receiver Operating Characteristic (ROC) curve analysis

As shown in Table 2 and Figure 1, the ROC curve analysis was used in the present study to compare the diagnostic values of serum oxidative stress biomarkers depending on the area under the ROC curve (AUC). The higher AUC corresponds to a better diagnostic test. Serum levels of MDA showed a significant AUC (96%),($p = 0.001$), with sensitivity (90%) and specificity (80%) at a cut-off value (13.81 nmol/ml). Serum levels of TAC showed a significant AUC (76.9%),($p = 0.005$), with sensitivity (85.7%) and specificity (57.7%) at a cut-off value (0.90 mM/L). Serum levels of oxidized glutathi-

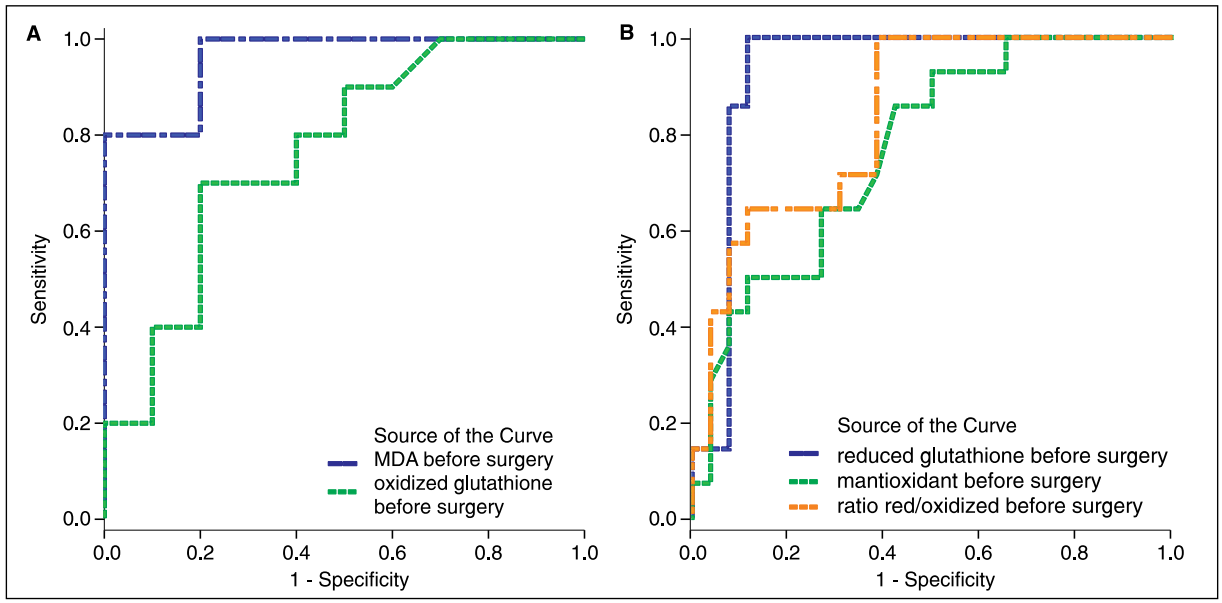

Figure 1. The ROC curve analysis of the assayed biomarkers for prediction of breast cancer

Table 2. The ROC curve-based characteristics for assayed serum markers in breast cancer patients before surgery

Variables	The area under the curve (%)	P-value	Cut-off value	Sensitivity %	Specificity %
MDA (nmol/ml)	96.0	0.001*	13.81	90	80
TAC (mM/l)	76.9	0.005*	0.90	85.7	57.7
GSSG (mg/ml)	70.5	0.035*	9.9	84.6	80.6
GSH (mg/ml)	92.9	0.000*	17.30	92.9	100
Glutathione Redox status GSH/GSSG))	82.1	0.001*	1.70	92.9	76.9

*Significance was considered at *p*-value < 0.05.

Table 3. The association of serum assayed biomarkers with the risk of breast cancer incidence

Assayed Biomarker		breast cancer group (n = 35)	control group (n = 35)	Odds ratio (OR)	95% CI	p-value
MDA (nmole/ml)	< 13.81 ®	13	30	10.15	1.69–11.06	P = 0.002 *
	≥ 13.81	22	5			
TAC (mM/l)	≥ 0.9 ®	5	20	8.00	2.51–25.51	P = 0.0004*
	< 0.9	30	15			
GSSG (mg/ml)	< 9.9®	5	28	24	6.82–84.43	P = 0.0011*
	≥ 9.9	30	7			
GSH (mg/ml)	≥ 17.30®	0	27	22.97	12.70–41.55	P = 0.0002*
	< 17.30	35	8			
Glutathione redox status (GSH/GSSG)	≥ 1.70 ®	2	32	17.60	7.56–23.11	P = 0.0001*
	< 1.70	33	3			

n — Sample size; ® — reference group; CI — Confidence interval;* — Significance was considered at *P*-value < 0.05

one showed a significant AUC (70.5),(p = 0.035), with sensitivity (84.6%) and specificity (80.6%) at a cut-off value (9.9 mg/dl). Serum levels of reduced glutathione showed a significant AUC (92.9),(p = 0.000), with sensitivity (92.9%) and specificity (100%) at a cut-off value (17.3 mg/dl). Serum levels of glutathione redox status (GSH/GSSG) showed a significant AUC (82.1), (p = 0.001), with sensitivity (92.9%) and specificity (76.9%) at a cut-off value (1.70).

The association of serum levels of oxidative stress biomarkers with the risk of breast cancer incidence

The values of odds ratio and confidence interval at certain cut-off values for serum levels of oxidative stress biomarkers were shown in Table 3.

Correlation of serum levels of oxidative stress biomarkers with breast cancer clinicopathological data

The results of the current study showed that none of the assayed biomarkers had a significant correlation with anyone of breast cancer clinicopathological features.

Discussion

Oxygen-free radicals (OFR), generated by a number of processes *in vivo*, are highly reactive and toxic [14]. However, biological systems have evolved an array of enzymatic and non-enzymatic antioxidant defence mechanisms to combat the deleterious effects of OFR. Reduced glutathione (GSH), in conjunction with glutathione peroxidase (GPx) and glutathione S-transferase (GST), plays a central role in the defence against free radicals, peroxides and a wide range of xenobiotics and carcinogens [15, 16].

Oxidative stress arises when there is an imbalance between OFR formation and scavenging by antioxidants. Excessive production of OFR can cause oxidative damage to biomolecules resulting in lipid peroxidation, mutagenesis and carcinogenesis. OFR-induced lipid peroxidation has been implicated in neoplastic transformation. Damage to the breast epithelium by OFR can lead to fibroblast proliferation, epithelial hyperplasia, cellular atypia and breast cancer [17].

In the current study, the serum levels of MDA were found to be significantly elevated in breast cancer patients than controls. As MDA is considered a marker for lipid peroxidation, this means that lipid peroxidation may play a role in breast carcinogenesis. Our results were in agreement with Rajneesh et al.,2008 [17].

Regarding the effect of MRM on the serum levels of MDA, the current study revealed significantly decreased levels of MDA after two weeks of surgery compared with its levels before surgery; this means that serum MDA can be used as an indicator for the effect of MRM on breast cancer patients. With respect to the effect of chemotherapy on the serum levels of MDA, our results showed a non-significant increase in serum MDA levels after 6 cycles of adjuvant combination chemotherapy compared with its levels after 2 weeks of surgery.

Total antioxidant capacity (TAC) measures the peroxyl-scavenging capacity of the extracellular antioxidant system that is comprised of sulphydryl groups, urate, ascorbate, carotenoids, retinol, α-tocopherol, bilirubin and proteins. TAC reflects residual antioxidant capacity

after the consumption of reactive oxygen species [18, 19]. In the current study, the serum levels of TAC were found to be significantly reduced in breast cancer patients before surgery than controls, this means that TAC may have a protective role against breast carcinogenesis. Our results supported those of Mahmood et al., 2009 [20] On the other hand, neither MRM nor chemotherapy has a significant effect on the serum levels of TAC.

Regarding the serum levels of GSH, the results of the current study showed significantly declined levels of GSH in breast cancer patients compared with normal healthy controls. This means that serum GSH can be used to differentiate breast cancer patients from controls. The results of the current study were in agreement with the findings of Yeh et al. [21] who reported that the levels of GSH were significantly decreased in the blood of breast cancer patients compared with controls. Yeh et al. [21] suggested that this could be due to both the increased GSH detoxification capacities, which can lead to GSH depletion within the red blood cells, and lower efficacy in the reduction of GSSG to GSH. These findings support the idea of the protective role of GSH against reactive oxygen species-mediated oxidative stress in cancer patients. At the same time, Yeh et al. [21] proposed that GSH should be regarded as an important biomarker for detecting breast malignancy.

With respect to the effect of surgery on serum levels of GSH, the results of the current study showed that the serum levels of GSH were significantly increased in breast cancer patients after two weeks of MRM compared with their levels before surgery. This means that the serum levels of GSH can be used to monitor the effect of MRM on breast cancer patients. The findings of the current study can be explained according to Navarro et al. [22] who suggested that the tumour mass acts as a source for peroxides that reacts with and consume GSH leading to depletion of GSH. On this base, removal of the tumour decreases these peroxides resulting in an increase in GSH content which is in agreement with the results of the current study. With reference to the effect of six cycles of chemotherapy, the results of the current study showed a non-significant effect of chemotherapy on the serum levels of GSH compared with their levels after two weeks of MRM. This means that GSH cannot be used to monitor the response of breast cancer patients to chemotherapy.

Regarding the serum levels of GSSG and the glutathione redox status (GSH/GSSG), the results of the current study indicated significantly elevated levels of GSSG and significantly decreased levels of glutathione redox status (GSH/GSSG) in the serum of breast cancer patients than controls. These findings were in accordance with the results of Navarro et al. [22] who reported significantly higher levels of GSSG and decreased levels of glutathione redox status (GSH/GSSG) in breast cancer patients compared with controls. In their study, Navarro et al. [22] postulated that during cancer growth, the glutathione redox status (GSH/GSSG) decreases in the blood due to an increase in GSSG levels. Two reasons may explain the increase in blood GSSG: (a) the increase in peroxide production by the tumour that can lead to GSH oxidation within the red blood cells; and (b) an increase of GSSG release from different tissues into the blood.

With respect to the effect of surgery on the serum levels of GSSG and glutathione redox status (GSH/GSSG), the results of the current study showed that the serum levels of GSSG were significantly decreased, with no significant effect on glutathione redox status (GSH/GSSG), in breast cancer patients after two weeks of MRM compared with their levels before surgery. This means that the serum levels of GSSG can be used to monitor the effect of MRM on breast cancer patients. The findings of the current study can be also explained according to Navarro et al. [22] who suggested that the tumour mass acts as a source for peroxides that reacts with GSH resulting in the formation of GSSG. When the tumour mass is removed the amount of these peroxides decreases resulting in a decrease in the amount of GSSG formed due to the detoxification reaction. With reference to the effect of six cycles of chemotherapy, the results of the current study showed non-significant effects of chemotherapy on the serum levels of GSSG and glutathione redox status (GSH/GSSG) compared with their levels after two weeks of MRM. This means that none of these biomarkers can be used to monitor the response of breast cancer patients to chemotherapy.

The significant elevation in the serum levels of MDA and GSSG and the significant decline in the serum level of TAC, GSH and glutathione redox status (GSH/GSSG) in breast cancer patients before surgery compared to normal controls suggested the possibility of using anyone of these biomarkers for prediction of breast cancer to differentiate the breast cancer patient from the normal healthy controls. This directed us to compare the diagnostic accuracy of these biomarkers to determine which of them has the highest and the lowest diagnostic value. This comparison was carried out using the ROC curve analysis in such a way that the greater area under the ROC curve corresponds to a better diagnostic test. Serum levels of MDA showed the greatest significant area under the curve (96%) followed by GSH (92.9%), glutathione redox status (GSH/GSSG) (82.1%), TAC (76.9%) then GSSG (70.5%). These results suggested that serum levels of MDA are superior to GSH, GSH/GSSG, TAC and GSSG for diagnosis of breast cancer patients. The results of the current study supported those reported by Pande et al. [23] who found that the diagnostic accuracy of serum MDA

was superior to TAC for distinguishing breast cancer patients from controls

Regarding the association of the assayed biomarkers with the risk of breast carcinogenesis, the results of the current study illustrated that increased levels of serum MDA and GSSG and decreased levels of TAC, GSH and glutathione redox status can significantly potentiate breast cells for malignancy. Pande at al., 2012 [23].

From the current study, it could be concluded that all of the assayed biomarkers have a diagnostic role in breast cancer patients with MDA and GSH being superior to the other biomarkers for discrimination of breast cancer patients from controls. Serum levels of MDA, GSH and GSSG were good indicators for monitoring the effect of surgery. All the assayed biomarkers were found to be associated with breast cancer risk. None of the assayed biomarkers could predict the effect of chemotherapy.

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VIA MEDICA

ORIGINAL ARTICLE

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Long-term survival in patients with NSCLC treated with single-fraction vs. multi-fraction palliative radiotherapy in the case of lung tumour, brain metastases and bone metastases

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ABSTRACT

Background: Patients with advanced non-small cell lung cancer (NSCLC) are candidates for different types of treatment, including chemotherapy and radiotherapy or supportive care. Despite the fatal prognosis in advanced disease, many experienced radiation oncologists will apply radiation at low doses with the intention of palliative care.

Methods: We used an extensive database of medical patients diagnosed with NSCLC, treated with palliative radiotherapy at the Oncology Centre in Bydgoszcz, from June 1998 to December 2013. A group of 3202 patients was divided into subgroups: Group A)1762 patients irradiated on the lung tumor (without distant metastases): Total dose: A1) 6Gy/1 fr. (n = 19); A2) 8Gy/1fr. (n = 276); A3) 20Gy/5fr. (n = 1349); A4) 30Gy/10fr.(n = 118). Group B) 548 patients irradiated on the central nervous system (CNS) metastases: B1) 20Gy/5fr. (n = 476); B2) 30Gy/10fr. (n = 72). Group C) 892 patients irradiated on the bone metastases: C1) 8Gy/1fr. (n = 452); C2) 10Gy/1fr. (n = 30); C3) 20Gy/5fr. (n = 341); C4) 30Gy/10fr. (n = 69).

Results: Patients with irradiation of a lung tumour: The longest OS was observed in the group of patients irradiated with doses of 20 Gy (76%) and 30 Gy (7%). Patients with irradiation of bone metastases: No significant differences in OS were observed between the employed fractionation regimens. Patients with irradiation of CNS metastases: The choice of a higher dose of radiation therapy did not demonstrate differences in median OS values compared to a lower dose.

Conclusions: The patients who were prescribed single fraction palliative radiotherapy did not have poorer prognoses or experience shorter survival than patients who were prescribed multi-fraction pRT in the case of lung tumour, brain metastases and bone metastases.

Key words: palliative radiotherapy, survival, NSCLC, single-fraction, multi-fraction

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INTRODUCTION

Non-small cell lung carcinoma (NSCLC) represents approx. 85% of cases of lung cancer, with more than half of the patients having distant metastases at diagnosis [1]. Patients with advanced NSCLC are candidates for various therapies, including chemotherapy [2–3], radiation therapy [4–5] and life-supporting therapy [6]. Despite the fatal prognosis in advanced disease, many experienced oncologists will apply low-dose radiation therapy with palliative intent. The choice of an effective radiation dose that will not cause significant complications remains discussible.

In our study, we conducted a retrospective analysis of the effectiveness of palliative radiation therapy in 3202 patients with advanced NSCLC using different methods of radiation dose fractionation depending on the location of lesions (lung tumour, bone metastases, CNS metastases).

Table 1. Patient characteristics

		Location of lesions		
	Number of patients (n = 3202)	**Lung**	**Bone**	**CNS**
Mean age	62.54 (22–93)	64.93 (22–93)	60.04 (30–89)	60.65 (36–87)
Sex (male/female)	2244/958	1300/462	596/296	348/200
Location of lesions	Number of patients			
Lung				
6 Gy	19 (1%)			
8 Gy	276 (16%)			
10 Gy	0			
20 Gy	1349 (76%)			
30 Gy	118 (7%)			
Bone				
6 Gy	0			
8 Gy	452 (51%)			
10 Gy	30 (3%)			
20 Gy	341 (38%)			
30 Gy	69 (8%)			
CNS				
6 Gy	0			
8 Gy	0			
10 Gy	0			
20 Gy	476 (87%)			
30 Gy	72 (13%)			

Material and methods

Patient database

An extensive medical database of patients treated at the Oncology Center in Bydgoszcz, Poland, was employed. The analysis included patients diagnosed with advanced NSCLC with no prior surgical intervention, treated with palliative radiation therapy between June 1998 and December 2013.

A group of 3202 patients was divided into three subgroups:

A) 1762 patients subjected to primary irradiation of a lung tumour (with no distant metastases revealed in imaging)

A-1) within this group, 658 patients received chemotherapy after radiation therapy

B) 548 patients subjected to primary irradiation of brain metastases

C) 892 patients subjected to irradiation of bone metastases

Data collected from all patients included: demographic data, symptoms, initial disease stage, prognostic factors, including weight loss and fitness, and therapeutic parameters of irradiation, including radiation dose, fractionation and total treatment time. Date of death was used as the data cut-off point. Every patient expressed and signed their informed consent to therapy with palliative intent. Detailed patient characteristics are presented in Table 1.

Treatment methods

Palliative radiation therapy was administered to all studied patients with non-small cell lung carcinoma. The areas of irradiation included lung tumour, metastatic bone tumour and metastases to the CNS. Doses ranging from 6 Gy to 30 Gy were administered with a different fractionation regimen (1–10 fractions) and total treatment time (1–10 days). Systemic treatment was offered to all patients with stage IV NSCLC.

The total number of patients undergoing palliative radiation therapy was 3202, including 1762 patients with irradiation of a lung tumour (without distant metastases), 548 patients with irradiation of brain metastases and 892 patients with irradiation of bone metastases.

Table 2. OS values for the three subgroups of patients depending on the dose of radiation therapy and the location of the treated metastases

		Lung		
Dose/Number of patients	**6 Gy (n = 19)**	**8 Gy (n = 276)**	**20 Gy (n = 1349)**	**30 Gy (n = 118)**
Median OS (months)	6	5	7	7
		Bone		
Dose/Number of patients	**8 Gy (n = 452)**	**10 Gy (n = 30)**	**20 Gy (n = 341)**	**30 Gy (n = 69)**
Median OS (months)	5	4	4	5
		CNS		
Dose/Number of patients	**–**	**–**	**20 Gy (n = 476)**	**30 Gy (n = 72)**
Median OS (months)	–	–	4	4

After the completion of radiation therapy, 100% of the treated patients of each group were followed-up for overall survival (OS). The three subgroups of patients were irradiated with different doses, which constituted a criterion of patient selection and follow-up for OS in these subgroups (Table 1).

Statistical analysis

The arithmetic mean and median OS was calculated using the Microsoft® Excel 2011 spreadsheet. Statistical significance of differences between the obtained results was checked using the logrank test ($p < 0.05$) in the STATISTICA software v. 13.0 by StatSoft. Comparative analysis of OS for all investigated groups was conducted using the Kaplan-Meyer method.

Results

Patients with irradiation of a lung tumour:

Median OS was 5–7 months after the completion of radiation therapy. The longest OS was observed in the group of patients irradiated with doses of 20 Gy (76%) and 30 Gy (7%). Patients who received lower single doses of 6 Gy (1%) and 8 Gy (16%) survived an insignificantly shorter period, but this could be caused by the small size of the group and the patients' individual disease characteristics (logrank test, $p = 0.06$) (Table 2, Fig. 1).

Patients with irradiation of bone metastases:

No significant differences in OS were observed between the employed fractionation regimens (Table 2, Fig. 2). The choice of a single radiation fraction for bone metastases not only resulted in a similar OS as with a higher dose administered in a few fractions but also allowed one-day treatment.

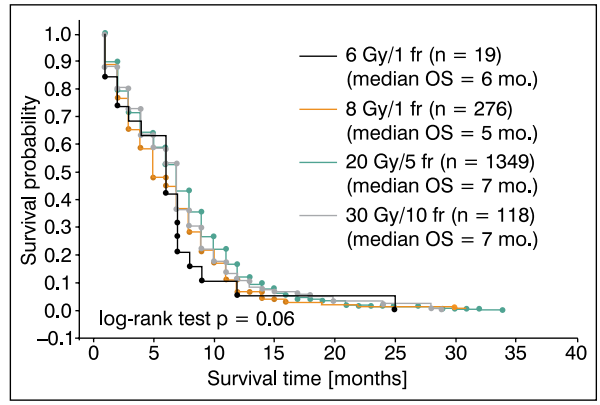

Figure 1. Kaplan-Meier survival curves for patients diagnosed with a lung tumor treated with palliative radiation therapy at different doses. Curves for each radiation dose have a very similar shape and course. The course is almost identical in the first 4 months and after the 14th month (most deaths occurred before the 14th month following the completion of radiation therapy). The small visible increase in the number of deaths of patients irradiated with the 6 Gy dose can be a result of a statistical error, as the group of patients irradiated with the 6 Gy dose is clearly smaller than the other groups. Individual patient characteristics could contribute to the results obtained in this small subgroup as well. In the 6th month, a large increase in the number of deaths in the group irradiated with the 30 Gy dose can be seen. The graphs for patients irradiated with the 8 and 20 Gy doses are very similar throughout their continuity, with minor deviations seen only between the 5th and the 6th month. Differences in the OS values for each subgroup of patients are statistically insignificant (logrank test, $p = 0.06$).

Patients with irradiation of CNS metastases:

The choice of a higher dose of radiation therapy did not demonstrate differences in median OS values

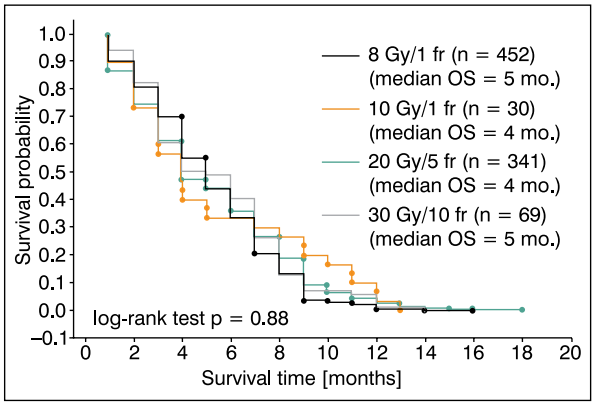

Figure 2. Kaplan-Meier survival curves for patients diagnosed with bone metastases of NSCLC treated with palliative radiation therapy at different doses. The course of four curves is comparable and illustrates the lack of a significant impact of dose level and fractionation on OS (logrank test, p = 0.88). Patients irradiated with the 10 Gy dose after the 4th month have a more flat curve than the other doses, with a proportionally lower death rate (a small number of deaths between the 4th and the 9th month). In the group of patients who received the 30 Gy dose, a sudden increase in the number of deaths in the 3rd month can be seen. Curves for the 8 and 20 Gy doses, the most commonly used doses in clinical practice, have a very similar course with minor deviations in each month. The similar course of curves for the groups irradiated with the 8 Gy dose in 1 fraction and the 20 Gy dose in 5 fractions (OS = 5 months) should lead to the choice of irradiation in a single dose in these clinical cases.

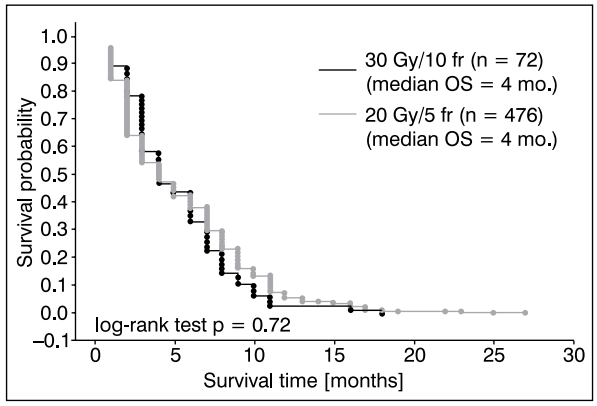

Figure 3. Kaplan-Meier survival curves for patients diagnosed with CNS metastases of NSCLC treated with palliative radiation therapy at different doses. Curves for both doses are very similar. In both cases, most deaths occurred in the first 4 months. In the subsequent months of follow-up, the patients' OS was similar (logrank test, p = 0.72).

compared to a lower dose. Based on the conducted analyses, it was found that increasing the total radiation dose does not extend the mean OS values (Table 2, Fig. 3).

Discussion

Palliative radiation therapy administered in a single dose allows quickly obtaining the desired effect without exposing patients to prolonged therapeutic procedures. Among the most common benefits of a single administration of a lower dose of radiation therapy in palliative treatment is its analgesic effect [7–8]. Often, bleeding from the upper respiratory ways in cases of tumours located in the lungs is stopped. The most frequent reason for palliative radiation therapy in patients with NSCLC is hemoptysis that can be stopped with low doses administered at appropriate intervals [9].

In a study by Ma et al., a meta-analysis of randomized, controlled trials involving a total of 1730 patients with locally advanced lung cancer was conducted [10]. The aim of this meta-analysis was to compare a higher (≥ 30 Gy) and lower (< 30 Gy) dose of radiation therapy in mitigating the symptoms and improving patient survival. The combined odds ratios (ORs) did not indicate a significant difference in the mitigation of cough, chest pain and hemoptysis between groups subjected to radiation therapy at the higher and the lower dose. OS values after the 1st and the 2nd year were similar between the high-dose and low-dose groups. In another retrospective study [11] involving 232 patients with NSCLC, the most frequently recommended dose was 2 fractions of 8.5 Gy (34%), followed by 10 fractions of 3 Gy or equivalent doses (30%, EQD2 approx. 33 Gy). OS was significantly shorter in the case of the 2 × 8.5 Gy regimen (median OS 2.5 months compared to 5.0 months for the 33 Gy regimen). In our study, a greater range of therapeutic doses was used, but comparative analysis revealed similar conclusions regarding the benefits of lower doses of radiation therapy in the palliative treatment of locally advanced lung cancer.

In the investigated subgroup of patients with irradiation of bone metastases, no significant differences in OS were found. The choice of a single irradiation with a dose of 8 Gy in 1 fraction for bone metastases not only offers a similar OS, but also allows for further sessions of radiation therapy for the treatment of recurrent osteoarticular pain or improvement of neurological signs in cases of spinal cord compression, as well as for an effective reduction of symptoms caused by compression of neighboring organs by the tumor, bleeding or infiltration [12–16].

The acknowledged regimen for treating CNS metastases is radiation therapy at 30 Gy in 10 fractions. In patients with poor prognosis, a smaller total dose of 20 Gy in 5 fractions is used [17].

In our study, the choice of the higher dose of radiation therapy did not demonstrate differences in OS compared to the lower dose (Fig. 3). Some researchers pointed out the doubtful results of reduced neurological symptoms after palliative radiation therapy of brain metastases [18–20]. However, a better understanding of both the acute and the chronic toxicity of whole-brain radiation therapy should lead to more selective use of this procedure.

Conclusions

The use of a single fraction of palliative radiation therapy allows patients to conduct a single comprehensive hospital visit and obtain a therapeutic and analgesic effect, as well as to optimize their quality of life without the need for further hospital visits. In relation to the studied groups, the analysis should include the treatment cost and travel expenses that are lower with a single hospital visit. Therefore, due to the specificity of patients in palliative care and the progression of the disease, the selected therapeutic regimens should be as short as possible but effective, along with a rationally selected radiation dose.

The study demonstrated that the clinical effect of using high-dose irradiation is comparable to that of multi-fraction radiation therapy in palliative treatment.

Disclosure of interest

The authors declare that they have no conflict of interest

Financial disclosures

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VIA MEDICA

ORIGINAL ARTICLE

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Recurrent Pneumonia in Children Admitted to Assiut University Children Hospital. Magnitude of the Problem and Possible Risk Factors

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ABSTRACT

Background: Incidence data indicate that recurrent pneumonia occurs in 7.7–9% of all children with community acquired pneumonia. We aim by this study to assess the prevalence of recurrent pneumonia among the admissions with diagnosis of pneumonia in Assiut university children hospital for one year and to try to define the possible related risk factors. Patients and methods: This is a prospective hospital based study in Assiut University Children's Hospital. Children younger than 16 years admitted with a hospital diagnosis of pneumonia to Assiut University Children's Hospital for one year from 1 February 2017 to 31 January 2018 were included.

Results: Approximately 1 in 12 children with pneumonia in our locality have recurrent pneumonia with percentage of 12.61%. Cardiac diseases especially congenital heart diseases was the cause among 25.45% of the cases, immunodeficiency diseases represented 20.9% of the cases ,while bronchial asthma was the cause of recurrent pneumonia among 16.36% of the cases. As regard to risk factors in studied group, prematurity was detected among 7.27 % of the cases while more than 50% were formula fed and more than 60% exposed to pollution. Father smoking was detected among 36.36% of cases. Patients aged > 6 years showed significantly higher frequency in risk factors as obesity, indoor and outdoor pollution and use of steroids in comparison to other age groups. However, patients aged 0-3 years exhibited significantly higher % frequency of having heart disease, oro-motor in coordination /swallowing dysfunction, gastro esophageal reflux and under nutrition as risk factors for recurrent pneumonia in comparison to other age groups.

Conclusions: The most frequent underlying cause for recurrent pneumonia in Assiut University Children's Hospital which presents the largest referral pediatric hospital in Upper Egypt for one year according to our study was cardiac diseases; the second most frequent cause was immunodeficiency diseases, followed by bronchial asthma. Risk factors for recurrent pneumonia include socio-economic status of studied cases, prematurity, exposure to passive smoking and in or outdoor pollution, obesity, under nutrition, lack of breast feeding, gastro esophageal reflux and steroids usage.

Key words: Recurrent pneumonia, community acquired pneumonia congenital heart disease, gastro esophageal reflux disease, immunodeficiency diseases, bronchial asthma, socio-economic status, chronic lung diseases, pulmonary tuberculosis , aspiration syndrome, anatomical congenital respiratory anomalies, cystic fibrosis.

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Introduction

Pneumonia is the top infectious killer of children under 5 years worldwide, resulting in 935,000 deaths each year. According to the "Global Action Plan for Prevention and Control of Pneumonia", around 20% of the child deaths that occurred globally in 2007 were caused by pneumonia, and more than 90% of these deaths were in developing countries [3]. In Egypt, it was estimated that 10% of children deaths below the age of 5 years is likely caused by pneumonia and other acute respiratory infections [4]. Egypt has around 2 million cases of children with pneumonia every year [5], and 42,000 Egyptian children under 5 die annually [6]. Ap-

proximately 6% of infants experience at least one episode of pneumonia during the first two years of life [7]. Recurrent pneumonia (RP) is defined as at least two episodes of pneumonia in one year or three episodes ever, with intercritical radiographic clearing of densities [8]. Incidence data indicate that RP occurs in 7.7–9% of all children with community acquired pneumonia (CAP) [1, 2].As a result, RP represents a frequent presenting manifestation in the general pediatric practice and is a very common reason for referral to pediatric chest physicians [1].

The challenge for the physician approaching RP is to discriminate between children with self-limiting or minor problems, that do not require a diagnostic work-up, and those with an underlying disease, for whom further investigation is mandatory. Therefore, determining which child should be investigated relies on clinical judgment, that should take into account the patient's history, the clinical course of the episode, and any symptoms and/or signs indicating the presence of an underlying disease [9].Most of the children hospitalized during the first episode of pneumonia had a known predisposing condition for pneumonia recurrence including neuromotor disorders with feeding problems, gastro-esophageal reflux ,congenital heart disease, asthma, airway malacia and vascular malformations [10].

The aim of the study is to assess the prevalence of recurrent pneumonia among the admissions with diagnosis of pneumonia in Assiut university children hospital for one year and to try to define the possible related risk factors.

Patient and methods

This is a prospective hospital based study in Assiut University Children's Hospital. Children younger than 16 years admitted with a hospital diagnosis of pneumonia to Assiut University Children's Hospital for one year from 1 February 2017 to 31 January 2018 were included.

All admissions to Assiut University Children Hospital were recorded over one year. Cases of pneumonia were identified and examined for criteria of recurrent pneumonia either on prospective bases.

Studied cases were subjected to the following:

History

Including age, sex, residence, neonatal history, socio-economic status for health research in Egypt (2012), duration of symptoms and treatment given, history suggestive of immunodeficiency (e.g. persistent diarrhea, cutaneous infections as boils, abscesses etc.). History of recurrent upper respiratory tract infections (defined as more than six serious URI with fever, cough, sore throat and running nose per year and history of pervious hospitalization, history of contact with tuberculosis patient, immunization status, developmental milestones and history of risk factors as prematurity, co morbid conditions as (heart diseases, GERD, oromotor or swallowing dysfunction) parental smokingand environmental pollution.

Clinical Examination

Complete physical examination including vital signs, anthropometric measurement and calculating body mass index (BMI) which equal weight divided by square of the body height in units of kg/m2 for children more than 2 years old, calculating weight for height, weight for age and height for age percentiles for children less than 2 years old for assessment of nutritional status. Also presence and degree (if present) of respiratory distress, clubbing and full chest and cardiac examination were assessed.

Investigations

All cases of recurrent pneumonia were subjected to the following investigations:

1. Pulse oxmeter assessment.
2. Chest X- ray.
3. Complete blood count with differential count.
4. C- reactive protein assay.
5. Mantous test (Tuberculin sensitivity test).
6. Echocardiography.

Moreover, some cases with recurrent pneumonia well be subjected

(According to every case individually based on history and clinical examination) to:

1. Computerized tomography of the chest in cases suspected of chronic lung diseases, anatomical congenital respiratory anomalies and suspected F.B inhalation.
2. Abdominal ultrasound and barium or gastro-graffin swallow with x-ray study in cases suspected gastro-esophageal reflux and recurrent aspiration.
3. Laryngoscopy or flexible fiber-optic bronchscopy in cases of recurrence of pneumonia on the same side, suspected laryngo or bronchomalacia and suspected F.B inhalation
4. Immunoglobulin quantitative assay, number of T and B lymphocytes and flow cytometric quantitative determination of leukocyte oxidative burst for children suspected to have immunodeficiency.
5. Cystic fibrosis DNA analysis for cases clinically suspicious having Cystic fibrosis.
6. Enzyme linked immunosorbant assay (ELISA) for human immunodeficiency virus (HIV) infection for suspected cases.

Inclusion criteria

Infants and children below 16 years old diagnosed on clinical and radiological grounds as recurrent

Table 1. Socio-economic status according to EL Sherbini Modified Score (2012)

Name of head of family: **Address:**

Definition of the family: It includes nuclear cc joint family Married couple with unmanied children or without children. Head of the family will be either husband/wife. Dependent father/mother/brother/sister does not become member of the family unless he/she is earning and one kitchen with pooled income is managed by him/her.

This scale includes 7 domains with a total score of 84.

Sodoeconomic level: to be classified into very low, low, middle and high levels depending on the quartiles of the score calculated.

NB In case of death or retirement of husband or wife, record the education and occupation before death or retirement

Education and cultural domain
(for both husband & wife)
(score — 30)

Highest level of education	Husband	Wife
Illiterate	0	0
Read & write	2	2
Primary	4	4
Preparatory	6	6
Secondary (general & technical of 3 or 5 years)	8	8
Intermediate (2 years) institutes	10	10
University graduate	12	12
Postgraduate degree	14	14

Access to health information (1 each for the following items): Printed materials, e.g. books, posters, booklets, etc.; Audiovisual message on television &/or radio

Occupation domain
(for both husband & wife)
(score — 10

Occupation	Husband	Wife
Non-working/housewife	0	0
Unskilled manual worker	1	1
Skilled manual worker/farmer	2	2
Trades/business	3	3
Semi-professional/clerk	4	4
Professional	5	5

Family possessions domain
(score — 12: 1 each for the presence of items given below)
Refrigerator — Radio — Television — Washing machine — Telephone/ mobile phone — Car — Agricultural land — Non-agricuhural land for housing — Shop or animal shed — Other house (beside the house in which the family is living) — Animals/poultry — Computer/ Internet

Family domain
(score —10)
Residence: Urban slum — 0; Rural -1; Urban — 2
Number of family members (parents, children & all dependents): < 5 members — 2; ≥ 5 members — 1
Number of earning family members:1 member — 1; 2 members — 2; ≥ 3 members — 3
Education of children (aged ≥ 5 years, whether free or private education): All children going or ever gone to school/university — 3; ≥ 50% going or ever gone to school/university — 2; <50% going or ever gone to school/university — 1; None go/gone to school/university/not applicable — 0

Home sanitation domain
(score — 12)
Services (1 each for the presence of the following items): Pure water supply — Electricity — Natural gas — Sewerage system — Municipal collection of solid wastes — Flush latrine — Air conditioning
Type of house: Owned, ≥ 4 rooms — 4; Owned, < 4 rooms — 3; Rented, ≥ 4 rooms — 2; Rented, < 4 rooms — 1; No place to reside — 0
Crowing index: (number of family members divided by number of rooms): ≤ 1 person per room — 1; > 1 person per room — 0

Economic domain
(score — 5)
Income from all sources: In debt — 0; 1 Just meet routine expenses — 1; Meet routine expenses and emergencies — 2; Able to save/invest money — 3
Family receives govemmental support: Yes — 1; No — 0
Family pays tax: Yes — 1; No — 0

Health care domain
(score — 5)
Usual source of health care: Private health facilities — 5; Health insurance — 4; Free governmental health service — 3; More than one of the above sources — 2 Traditional healer/self care — 1

pneumonia. Recurrence is evidenced by the presence of documented previous episodes of pneumonia, 2 or more per year or 3 or more episodes in life time based on radiological evidence of two chest x rays with diagnosis of pneumonia with completely resolved chest x ray from pneumonia in-between.

Exclusion criteria

Age more than 16 years old , lack of radiographic evidence of pneumonia , lack of radiographic evidence of definite recurrence (for recurrent pneumonia cases) and cases of non resolving or persistent pneumonia. Patients with obvious neurological diseases that lead to recurrent pneumonia also were excluded.

Patient clinically diagnosed as recurrent pneumonia, and those with non recurrent pneumonia will be subjected to x-ray examination for radiographic evidence of pneumonia .Only patient with radiographic evidence of pneumonia will be continued to be investigated.

Results

This study was done in Assiut University Children Hospital for one year from 1 February 2017 to 31 Janu-

ary 2018 .All cases with pneumonia admitted to all units in the hospital have pneumonia were (872) case and (110) cases fulfilled the inclusion criteria of recurrent pneumonia with percentage of 12.61%.

Investigations were done:

— Mantous test was done for tuberculin sensitivity using 1T purified protein derivative (PPD) which was administered intradermally and reading was taken after 48-72 hours for all cases which included as recurrent pneumonia in the study. It was found that five cases had positive tuberculin testing. Gastric lavage and sputum study for acid fast bacilli were positive in two cases.

— Echocardiography was done to all cases included in the study and have recurrent pneumonia and twenty eight cases were found to have congenital heart disease.

— Gastro — esophageal reflux disease (GERD) was diagnosed in four children by clinical suspicion and confirmed by barium swallow.

— Abdominal ultrasound was done for all cases with clinically palpable organomegaly, cases with gastro esophageal reflux disease and for all cases with suspected to have immunodeficiency.

— Flexible fiberoptic bronchoscopy was performed for three cases with radiological evidence of recurrent atelectasis in same side showing chronic inflammatory process and for two cases diagnosed as bronchiectasis and two cases diagnosed as vascular ring.

— C.T scanning for chest examination was done for nine cases with atelectasis and lobar pneumonia and for all cases with chronic lung disease (Bronchiectasis, Interstitial lung fibrosis (IPF).

— Cystic fibrosis DNA analysis also shows only one case with Cystic fibrosis from clinically suspicious included patients.

Table 2. Demographic data of studied cases with recurrent pneumonia.

Item	Descriptive "n=110"
1. Gender:	
• Male	72(65.45%)
• Female	38(34.54%)
2. Age "years"	
Mean±SE	3.91±0.40
(min-max)	(0.2-16)
• 0–3 yrs.	69(62.72%)
• > 3–6 yrs.	15(13.63%)
• > 6 yrs.	26(23.63%)
3. Residence:	
• Rural	25(22.73%)
• Urban	85(77.27%)

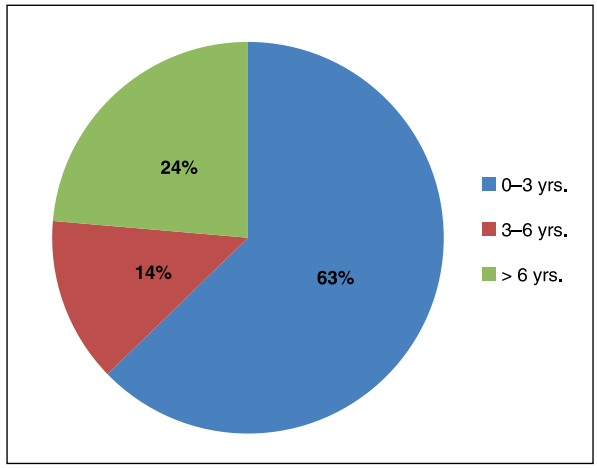

Figure 1. Distribution of age (years) among the studied patients.

— Enzyme linked immunosorbant assay (ELISA) for human immunodeficiency virus (HIV) infection to suspected five cases but all results were negative.

— Seven cases with recurrent pneumonia, the etiology of recurrence was unknown inspite of most of previous investigations were done to them.

Table 2 and figure 1 show demographic data of the studied cases. It was found that (65.5%) of cases were males. The age ranged from 0.2-16 years with mean age 3.91 ± 0.40 years. The majority of the included patients were belonged to age group 0–3 years (62.72%). About (77.27%) of studied cases was from urban areas.

Table 3 shows socio-economic status of studied cases according to El Sherbini modified classification Score, (2012). According to maternal domain about half of mothers read & write (54.54%). About (77.2%) of studied cases were from urban areas. Regarding occupation parents domain, there were (54.54%) of the parents were skilled manual worker. About family possessions domains there were (59.09%) had score 7. In economic domain the majority of cases (84.54%) had met routine expenses &emergencies. Also all cases had number of family members more than 5 members. Regarding crowding index there were about (94.5%) of cases had more than one person/room. According to El Sherbini modified score (48.18%) of the families belonged to middle class, while (43.64%) were classified as low class.

Table 4 shows history in the included patients. The mean value of disease duration was 11.84 ± 3.29 days. There was history of cough among (94.54 %) of the patients, while (56.36%) had history of fever and (50.0%) had recurrent upper respiratory tract infection. About (22.72%) cases had history of atopy/allergy and about (11.81%) of cases had history of immuno-deficiency from first months of age.

Table 5 shows descriptive data of investigations were done to the studied group including pulse oximeter, C - reactive protein and complete blood count with differential count.

Table 6 and figure 2 show causes of recurrent pneumonia. The patients with cardiac disorders showed the highest % frequency of cases of recurrent pneumonia (25.45%), followed by (20.9%) those with immuno-deficiency, while (16.36%) of cases were due to bronchial asthma. However, (6.36%) of the cases, the etiology was unknown.

Table 7 shows classification of cardiac causes of recurrent pneumonia. Patients with cyanotic heart diseases diagnosed as TGA (17.8%) of the cases, while (28.7%) of cardiac patients with a cyanotic heart diseases had ventricular septal defect.

Table 8 among 23 cases diagnosed with immunodeficiency, (52.17%) were due to B-cell deficiency, while (26.08%) were diagnosed as phagocytic cell defect.

Table 9 shows risk factors in study group. Prematurity was detected among (7.27%) of the cases while more than 50% were formula fed and more than 60%

Table 3. Socio-economic status of studied cases according to ElSherbini modified Score (2012) in Egypt.

Item	Descriptive "n=110"
1. According to maternal education domain:	60 (54.54%)
• Read & write	32 (29.29%)
• Preparatory	18 (16.36%)
• Secondary	
2. Family Domain:	85 (77.27%)
• Urban	25 (22.72%)
• Rural	
3. Occupation parents domain:	30 (27.27%)
• Unskilled manual worker	60 (54.54%)
• Skilled manual worker	10 (9.09%)
• Semi-Professional	0.0
• Professional	
4. Family possessions domains:	62 (59.09%)
• Score 7	33 (30.0%)
• Score 5-6	15 (13.63%)
• Score <4	
5. Economic domain:	
• In debt	
• Just meet routine expenses	0.0
• Meet routine expenses &emergencies	3 (2.70%)
• Able to save money	93 (84.54%)
6. Number of family members:	14 (12.72%)
• < 5 members	
• ≥ 5members	0.0
7. Crowding index" "number of family member/divided by number of rooms":	110 (100%)
• ≤ person/room=1	
• > 1person/room	6 (5.45%)
8. Home sanitation domain:	104 (94.5%)
• Score 4	
• Score 2	61 (55.45%)
9. Elsherbini modified score:	49 (4.54%)
• Very low	
• Low	3 (2.73%)
• Middle	48 (43.64%)
• High	53 (48.18%)
	6 (5.45%)

Table 4. History analysis in the studied group.

Item	Descriptive "n = 110"
1. Duration of disease "days"	11.84 ± 3.29
2. Cough	104 (94.54%)
3. Fever	62 (56.36%)
4. Wheezing	20 (18.18%)
5. Chronic rhino-sinusitis with post-nasal drip	00
6. Recurrent upper respiratory tract infections	55 (50.0%)
7. Atopy/allergy	25 (22.72%)
8. Asthma	19 (17.27%)
9. Unexplained death, severe infections or multisystem diseases in family.	8 (7.27%)
10. Unusual organism or feature of systemic immunodeficiency	00
11. History suggesting Immuno-deficiency from first months of age	13 (11.81%)
12. Continuous, unremitting or worsening symptoms	10 (9.09%)

Table 5. Investigations done for the studied group.

Item	0-3yrs. "n = 69"	> 3-6yrs. "n = 15"	> 6yrs. "n = 26"
1-Pulse oximetry"%"	94.05 ± 0.99	92.60 ± 0.91	96.11 ± 2.26
CBC:			
2-WBCs "Cell/mm^3"	11.64 ± 6.42	13.04 ± 3.47	13.79 ± 4.97
3-Eosinophil "Cell/mm^3"	2.45 ± 0.39	4.83 ± 0.91	3.38 ± 0.67
4-Hb "g/dL"	10.05 ± 1.55	11.42 ± 1.60	11.31 ± 1.52
5-MCV "FL"	70.53 ± 13.35	76.44 ± 3.89	70.41 ± 16.34
6-MCH "pg"	25.04 ± 8.84	27.09 ± 4.38	25.52 ± 3.85
7-Platelets "10^3/uL"	393.26 ± 185.56	364.40 ± 78.43	308.30 ± 88.20
8-CRP "mg/dL"	36.30 ± 22.42	38.61 ± 22.41	42.32 ± 36.73

Table 6. Causes of recurrent pneumonia in the studied cases.

Item	Descriptive "n = 110"
1. Cardiac diseases	28 (25.45%)
2. Immuno-deficiency	23 (20.90%)
3. Bronchial asthma	18 (16.36%)
4. Chronic lung diseases	15 (13.63%)
• Bronchiectasis	7 (6.36%)
• Interstitial lung disease	8 (7.27%)
5. Aspiration syndrome:	7 (6.36%)
• GERD	4 (3.63%)
• Aspiration pneumonia	3 (2.72%)
6. Unknown	7 (6.36%)
7. Tuberculosis	7 (6.36%)
8. Anatomical congenital respiratory anomalies:	4 (3.63%)
• Tracho-esophgeal fistula	1 (0.90%)
• Vascular ring	2 (1.81%)
• Cleft palate	1 (0.90%)
9. Cystic fibrosis	1 (0.90%)

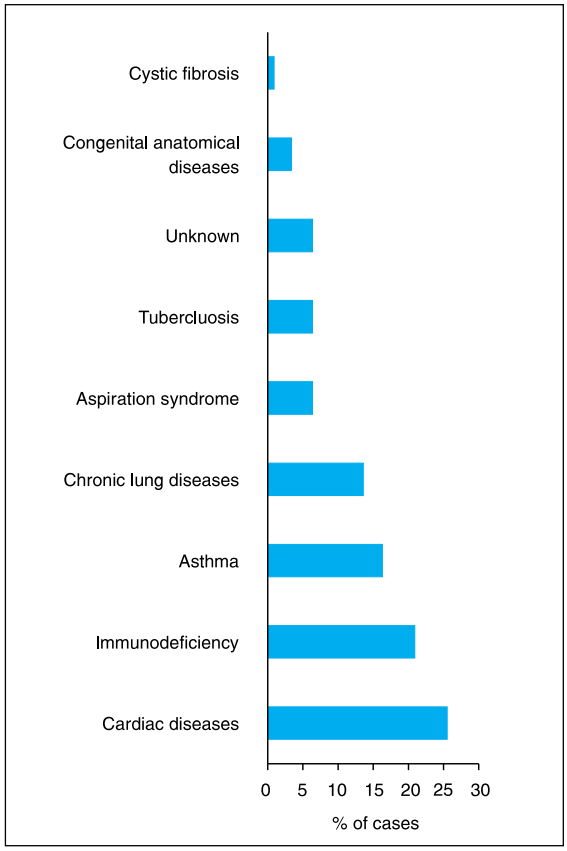

Figure 2. Causes of recurrent pneumonia among the studied patients.

Table 7. Classification of cardiac causes among patients with recurrent pneumonia.

Item	"N=28"
A. Cyanotic heart disease:	
1. *With decreased pulmonary flow:*	
— Double outlet Rt. Ventricle (with pulmonary stenosis).	1 (3.57%)
2. With increased pulmonary flow:	
— Transposition of great arteries.	5 (17.85%)
— Total anomalous pulmonary venous drainage (without pulmonary stenosis).	3 (10.71%)
B-A cyanotic heart diseases:	
1. ventricular septal defect.	8 (28.71%)
2. Atrial septal defect	4 (14.28%)
3. Atrio-ventricular septal defect	2 (7.14%)
4. Patent ductus arterious.	5 (17.85%)
5. Pink Fallot's tetrology.	2 (7.14%)

Table 8. Classification of causes of immunodeficiency among patients presented with recurrent pneumonia.

Item	"N = 23"
• Phagocytic cell defect"chronic granulomatous defect disease".	6 (26.08%)
• T-cell deficiency	2 (8.69%)
• B-cell deficiency	12 (52.17%)
• Combined T,B cell deficiency	3 (13.04%)

Table 9. Risk factors 0f recurrent pneumonia in the studied group.

Item	Descriptive "n = 110"
1. Prematurity	8 (7.27%)
2. Feeding history(4. 6months):	
Breast feeding	53 (48.18%)
Formula fed	57 (51.81%)
3. Co.morbid conditions:	
Heart disease	30 (27.27%)
Oro-motor incoordination/ swallowing dysfunction	7 (6.36%)
Primary or acquired immunodeficiency	
GERD	10 (9.09%)
Overweight/obesity	8 (7.27%)
4. Smoking (father)	7 (6.36%)
5. Respiratory disease/previous allergy	40 (36.36%)
6. Vaccinations:	31 (28.18%)
T.B	108 (98.2%)
Influenza	10 (9.09%)
Measles	106 (96.36%)
7. Use steroid	9 (8.18%)
8. Indoor pollution	42 (38.18%)
9. Outdoor pollution	38 (34.54%)
10. undernutrition	21 (19.09%)

exposed to pollution. Regarding vaccination for T.B and measles it was found that more than 95% of cases received these vaccines. Father smoking was detected among (36.36%) of cases.

Table 10 shows risk factors in relation to age groups among the studied cases. Patients aged > 6 years showed significantly higher frequency in risk factors as obesity, indoor and outdoor pollution and use of

Table 10. Risk factors of recurrent pneumonia in relation to age groups among the studied cases.

Item	0–3yrs. "n = 69"	> 3–6yrs. "n = 15"	> 6yrs. "n = 26"	P-value
1. Prematurity	12 (17.39%)	3 (20.0%)	2 (7.69%)	P = 0.442n.s
2. Feeding history(4.6months):				
Breast feeding	35 (50.72%)	6 (40.0%)	12 (46.15%)	P = 0.732n.s
Formula fed	34 (49.27%)	9 (60.0%)	14 (53.84%)	
3. Co-morbid:				P < 0.006
Heart disease	26 (37.68 %)	1 (6.66%)	3 (11.53%)	P = 0.108n.s
Oro-motor incoordination/swallowing dysfunction	7 (10.14%)	0	0	
Primaryor acquired immunodeficiency	9 (13.04%)	8 (53.33%)	6 (23.07%)	P = 0.135n.s
GERD				
Overweight/obesity	4 (3.63%)	0	0	P = 0.077n.s
4. Smoking in parents:	0	1 (6.66%)	6 (23.07%)	P < 0.000
5. Respiratordisease/previous allergy	16 (23.9%)	7 (46.7%)	17 (65.4%)	P < 0.001
6. Vaccinations:	22 (31.88%)	5 (33.3%)	4 (15.38%)	P < 0.001
T.B	68 (98.6%)	(100%)	5 (96.2%)	P = 0.628n.s
Influenza	0	3 (20%)	7 (26.9%)	P < 0.03
Measles	67 (97.9%)	15 (100%)	26 (95.7%)	P = 0.493n.s
8. Use steroid	2 (2.9%)	0	7 (26.9%)	P < 0.01
9. Indoor pollution	23 (33.33%)	5 (33.33%)	14 (53.84%)	P < 0.01
10. Outdoor pollution	19 (27.53%)	4 (26.67%)	15 (57.69%)	P < 0.04
11. undernutrition	16 (23.18%)	3 (20.0%)	2 (7.69%)	P < 0.001

steroids in comparison to other age groups. However, patients aged 0-3 years exhibited significantly higher % frequency of having heart disease, oro-motor incoordination/swallowing dysfunction, GERD and undernutrition as risk factors for recurrent pneumonia in comparison to other age groups.

Discussion

In present study, pneumonia was diagnosed in 872 of admitted cases and 110 cases fulfilled the inclusion criteria of recurrent pneumonia 12.61%. This agrees with Saad and his colleges (2013)(11) who found in previous study in Assiut in Upper Egypt that 9.2% of patients with pneumonia met the definition of recurrent/persistent pneumonia, 7% for recurrent and 2.2% for persistent pneumonia .Also Morcos and his colleges (2016)(12) reported that 12% had recurrent pneumonia in El Galaa Teaching Hospital in Egypt. Similarly; 1–9% of patients met the criteria for recurrent pneumonia in previous studies (13, 14). However, other studies, Bolursaz et al (2017) (15) reported prevalence of 34.64 % of RP among patients with pneumonia in Tahran, Iran.

In present study, 65.5% of cases were males. This agrees with previous study done in Assiut University Hospital by Saad et al (2013)(11) as male patients represent 65% and female represents 35% of included RP cases. Bolursaz et al (2017)(15) also found that 55.0% of cases were males in Iran, that can be explained as male patients are more vulnerable to infections than female, also the tradition of favoring male to female in the community makes parents seek medical advice for male children earlier and more frequent. The majority of the included patients were belonged to age group 0-3 years (62.7%). This agrees with Bolursaz et al (2017) (15) who reported that 1% had onset of symptoms before 3 months of age, 6% between 3 and 12 months, 65% between 1 and 5years and 28% after the age of 5years, that can be explained as the age group 0-3 years had significantly higher % frequency of presence of co morbid conditions as congenital anomalies, congenital heart disease and under nutrition.77.27% of included cases were from urban areas, while 22.72% of included RP cases were from rural areas. This agrees with Li and his colleges (2017) (16) who reported that incidence of childhood pneumonia generally higher in urban areas with percentage of 73.45% for urban residents versus 26.55% for rural areas.

In the present study, there was history of cough among 94.45% of the patients, and 56.36% had history of fever while 50.0% had history of recurrent upper respiratory tract infection .Less than third of cases 22.72% had history of atopy/allergy and about 11.81% of cases had history of immuno-deficiency from first months of age. This agrees with Çapanoglu et al (2017) (17) who reported the most common presenting symptom was cough 95.5%, followed by fever 59.9% and wheezing 47.9%.

Our results are in line with Çapanoglu et al (2017) (17) who reported congenital heart diseases were the

most important cause for recurrent pneumonia in 33.9% of children with RP. Dilated blood vessels or chambers of the heart may compress the bronchi, causing impaired drainage of pulmonary segments. Also patients with congenital lesions causing left-to-right shunting and an increased pulmonary blood flow have an increased susceptibility to respiratory infections. Previous studies have reported congenital heart disease in 1.2–25.4 % of cases (10, 13,14). A left-to-right shunt can adversely affect lung function, and superimposed lower respiratory tract infections cause additional compromise and might lead to respiratory failure (18). In agreement with these figures, our results demonstrated that congenital heart diseases was identified among 25.45% of cases and TGA was diagnosed in 17.85% of the cardiac cases, while 28.7% of cardiac patients with a cyanotic heart diseases had ventricular septal defect.

In present study, 6.36 % of studied cases were due to aspiration syndrome, 3.36% with GERD and 2.72% with aspiration pneumonia (in spite of patients with obvious neurological diseases that lead to recurrent pneumonia were excluded from our study). Gastro-esophageal reflux disease was diagnosed in four children by clinical suspicion and confirmed by barium swallow. This agrees with Owayed et al (2000) (10) who reported that in developed countries, aspiration is considered an important cause of RP and could be due to gastro-esophageal reflux disease (GERD) or due to pharyngeal in coordination. Reflux should be confirmed by esophageal P^{Hh} study, while video fluoroscopy should be used to confirm in coordination. Technetium scan, esophagoscopy and biopsy may be used if P^{Hh} study is not conclusive, although in many cases the history would be sufficient (19).

In present study, 20.9% of studied cases proved to have immune-deficiency diseases. 52.17% of them were due to B -cell defect, 26.08% with chronic granulomtous disease, 13.04% due to combined T and B cell deficiency and 8.69% due to T cell defect. This agrees with Patria & Esposito (2013) and Hoving & Brand (2013) (2, 18) who reported that immune deficiency disorders were present among 7.7–17.75 % of cases of RP. Children with immune defects usually present with highly recurrent and/or severe bacterial infections of the respiratory tract without any seasonality, recurrent gastrointestinal infections and recurrent skin infections. The family history of immunodeficiency is often characterized by recurrent infections and early deaths. There is often a delay of years between the onset of symptoms and the diagnosis being made: this delay increases the risk of bronchiectasis and irreversible lung damage occurring before appropriate treatment is given (20).

In present study, cases with RP have asthma represented 16.36% of studied cases. This agrees with Douglas & Couriel (2001) (21) who reported asthma was diagnosed among 9.7% of studied patients. Asthma is the most important underlying illness for recurrent pneumonia in children reported by different researchers accounting for 15%–69 % of cases (14, 22, 23).The notion that asthma in children can be complicated by recurrent pneumonia has a long history and contributes to the confusion when assessing these children. This is especially true when the RML is involved. The right middle lobe syndrome is subject to atelectasis because of the anatomy of the bronchus and the lack of collateral ventilation with other lobes. Excess mucus production in hypersecretory asthma can lead to RML atelectasis. It is well recognized that the most common cause of the so-called 'right middle lobe syndrome' is asthma (24). Atopy was considered as an important risk factor for RP due to defective innate immune response of epithelial cells and interleukin 13-dependant reduced mucociliary clearance (25, 26). Allergic inflammation may also suppress the interferon response of innate immunity under certain circumstances (27).In contrast to our study and previous studies, Hoving & Brand (2013) (2) reported that asthma was not diagnosed as an underlying cause of recurrent pneumonia in their study. They believed that asthma is a rare cause of recurrent pneumonia in children, and if occurs this seems to be confined to very unusual and complicated cases of asthma.

Tuberculosis is one of the most common infectious diseases among children in the world. TB is suspected when an ill child has a history of chronic illness of usually more than 3 weeks of duration that includes cough and fever, weight loss or failure to thrive (28). Tuberculosis is a common cause of extra luminal compression of the airways associated with recurrent lung infections (18). In the present study, 6.36% of cases RP was due to tuberculosis. This agrees with Lodha et al (2002) (29) who reported pulmonary TB as a cause of recurrent pneumonia in 7.1% of patients. In addition, **Ç**elebi (2010) and his colleagues (30) reported TB in 4.8% of the cases. The relatively low percentage frequency of TB as a cause for RP in our study may be suggested by the high rate of vaccination 98.6% among our studied cases. In previous studies with recurrent pneumonia, (13, 22) reported pulmonary TB as a cause in 19.2% and 31.5% of patients, respectively. This relatively high prevalence of pulmonary TB should alarm the physicians and health authorities to take more intensive measures for prevention and control of this disease in these areas.

The present study demonstrated that, 13.63% of studied cases have chronic lung diseases, 7.27% with interstitial lung disease while bronchiectasis represented 6.36% of included cases. These results matching with recent hypothesis that RP early in life is a major risk factor for bronchiectasis (31), but only some children actually develop bronchiectasis after the first episodes of pneumonia. The early identification of

the patients at the highest risk of bronchiectasis could allow a diagnosis to be made when the bronchial wall lesions are still mild, thus favoring the implementation of appropriate preventive and therapeutic measures, and a better final prognosis (32).The development of bronchiectasis is a chronic, progressive pulmonary disorder characterized by the permanent dilatation of one or more bronchi due to structural modifications in the bronchial wall (33). In pediatric patients, bronchiectasis causes an accelerated decline in lung function that leads to repeated hospital admissions due to acute infectious exacerbations, a poorer quality of life, and possible premature death in early adult life (34). Recurrent pneumonia was considered as a risk factor for development of bronchiectasis in 6.7%- 8.5% of cases (1, 10).

In the present study, 4 patients 3.63% had congenital anatomical anomalies of the respiratory tract one patient had tracho-esophageal fistula, 2 had congenital vascular ring and one had cleft palate. Previous studies have reported congenital anomalies of the respiratory tract in 3.7-8.5% of cases (2, 10, 29). Recurrent chest infections are often the presenting feature of congenital abnormalities of the airways, lung parenchyma and pulmonary vasculature. For example, repeated episodes pneumonia are often the presenting feature of lobar sequestration, bronchial stenosis and bronchomalacia,and cystic adenomatoid malformations of the lung (35). Such an abnormality should be suspected if one lobe is repeatedly infected or if there is there is incomplete resolution after treatment. Computerized tomography and magnetic resonance scanning are helpful in defining the anomaly prior to surgical excision (36).

In our study the etiology of recurrent pneumonia was not recognized in 6.36% of studied cases in spite of careful history obtained and full investigation done to these cases. This agrees with Çapanoglu et al (2017) (17) who mentioned that the underlying cause of recurrent pneumonia was no specific in 16.3% of studied cases and Saad and his colleges (2013) (11) who mentioned also that in 10-20%of studied cases the etiology was unknown.

The current study showed that only one case had cystic fibrosis, diagnosed by cystic fibrosis DNA analysis .Limited data are available regarding CF prevalence among Egyptian children's has been believed to occur infrequently in Egypt; only few papers suggested its presence (37, 38).A history of neonatal jaundice, poor weight gain, steatorrhea and highly recurrent pneumonia may suggest cystic fibrosis, although atypical cases may present with recurrent pneumonia alone, in the absence of malabsorption. Also recovery of pseudomonas aeruginosa from the respiratory tract, especially the mucoid form, is highly suggestive of CF (18, 39).

In present study, according to El Sherbini modified score (2012) 48.18% of the families belonged to middle class, while 43.63%were classified as low class. This agrees with Wonodi et al (2012); Çapanoglu et al (2017) (17,40) who reported risk factors associating with RP in children especially poor socioeconomic status which represent an important risk factor commonly associated with increase the frequency of illness episodes in children .

Also high crowding index according to El Sherbini modified score (2012) was detected among 94.5% which represent important risk factor for recurrent pneumonia mostly due to increased exposure to respiratory pathogens (41).The definition of household crowding varies greatly (42). Household crowding was defined as two or more individuals sleeping in the same room as the child (43) or more 7 persons per household (44). Our results match with Çapanoglu et al (2017) (17) who reported overcrowded household as a definitive risk factor of RP.

In our study, we also found that 54.4% of mothers of studied cases only read and write and 29.29% of them had preparatory level of education. This agrees with De Souza et al (2000) (45) who mentioned that mother's education level has an undeniable and important impact on their children's health and understanding of them of the importance of the disease, its severity and use of health services. Also Nirmolia et al (2018) (46) showed lack of maternal education is significantly associated with occurrence of pneumonia.

History of prematurity was detected among 7.27% of studied cases. This agrees with Çapanoglu and his colleges (2017) (17) who mentioned prematurity as an important risk factor for pneumonia recurrence in children .The two main mechanisms that children with low birth weight put at risk of respiratory infections include low immunity level and defects in lung function. Also, these children also have limited iron, zinc and copper resources (47).Other studies that have been conducted in developing countries, showed the relationship between birth weight and infant mortality due to pneumonia or acute infection of the lower respiratory tract (48).Yoon et al (1997) (49) mentioned that a strong correlation exists between decreased mortality from pneumonia with increasing birth weight.

In the present study, 36.36% of studied cases had history of parental smoking which becomes one of the important risk factors for RP. This agrees with Çapanoglu et al (2017) (17) who reported that exposure to smoking presented in 57.1% of studied cases with RP. Passive smoking is a risk factor for developing respiratory tract infections in children (50). Passive smoking in children leads to suppression of phagocytic function and cilia cell activity, increase the likelihood of adherence of bacteria to the epithelium of respiratory tract (51).

According to our study, 19.09% of studied cases have under malnutrition. This agrees with most of

studies done before in the developing countries which reported strong correlation between under malnutrition and pediatric recurrent infections especially respiratory tract infections (51).Children with malnutrition have deficient immune responses, consequently these childhood infections are more severe in these children. Studies show children who their weight is less than 70% appropriate weight for their age compared to other children, increased an 8 times risk of mortality from pneumonia for them (48).

In present study 51.8% of studied cases were formula fed and only 48.2% were exclusive breast fed .This agrees with Çapanoglu et al (2017) (17) who mentioned that insufficient breastfeeding was one of the definitive risk factors strongly associated with increasing the frequency of RP. Ulshen (2005) (52) reported that breastfeeding can protect children against the risk of lower respiratory infections. In fact, breast milk gives passive protection against pathogens even with change of age infants (53).Complete or partial breast-feeding resulted in a 50% reduction in mortality from acute respiratory tract infections in children fewer than 18 months (54).

In present study 38.18% of cases with recurrent pneumonia exposed to indoor air pollution .There are documentations that the risk of pneumonia is enhanced following exposure to unprocessed solid fuel use by a factor of 1.8 (55) and also there is significant association between ARI and indoor air pollution (56). Nirmolia et al (2018) (46) have documented a significant association between indoor air pollution and pneumonia. It has been recommended that prevention of indoor air pollution from burning of solid fuel to switching over to better quality fuel, improved ventilation or some other measures will substantially reduce morbidity and mortality from pneumonia (55).This agrees with Po, et al (2011) ; Searing and Rabinovitch (2011) (57, 58) who reported that both indoor and outdoor pollution are a definitive risk factors for RP. Dherani et al (2008) (55) also reported that indoor air pollution has been determined to elevate the risk of pneumonia in children by approximately 80%.

Conclusion

1-Approximately 1 in 12 children with pneumonia in our locality have recurrent pneumonia with percentage of 12.61%.The most frequent underlying cause for recurrent pneumonia in Assiut University Children's Hospital which present the largest referral pediatric hospital in Upper Egypt for one year according to our study was cardiac diseases especially congenital heart diseases 25.45% , the second most frequent cause was immunodeficiency diseases 20.9% followed by bronchial asthma 16.36%. Other causes include chronic lung diseases, pulmonary T.B, aspiration syndrome, anatomical congenital respiratory anomalies and cystic fibrosis arranged respectively, while the etiology of recurrence was unknown in 6.36 % of the cases.

2-Prematurity was detected among 7.27 % of the cases while more than 50% were formula fed and more than 60% exposed to pollution. Father smoking was detected among 36.36% of cases. Patients aged > 6 years showed significantly higher frequency in risk factors as obesity, indoor and outdoor pollution and use of steroids in comparison to other age groups. However, patients aged 0–3 years exhibited significantly higher % frequency of having heart disease, oro-motor in coordination /swallowing dysfunction, GERD and under nutrition as risk factors for recurrent pneumonia in comparison to other age groups.

Finally, we hope that this study will help the pediatricians identify and hence prevent and manage the most common etiologies of recurrent pneumonia in our locality. Determining which case should be investigated relies on clinical judgment, depending on a careful history and physical examination, whether the child is improving clinically and whether there is any feature suggestive of an underlying condition. Early treatment of the child's underlying condition is crucial in order to stabilize lung disease and thus prevent progressive deterioration of most pulmonary diseases.

A wide range of tests is available and there is no evidence base to guide the clinician on the most appropriate timing or sequence of investigations. Ideally, the diagnosis should be confirmed or excluded with the minimum number of the least-invasive tests. The economic burden of an extensive diagnostic work-up should always be kept in mind. Choosing the most appropriate investigations for recurrent pneumonia should be individualized for every case separately according to detailed history and full clinical examination for each case.

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VIA MEDICA

ORIGINAL ARTICLE

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Effect of commercially available spices and herbs on the survival of *Listeria monocytogenes* and *Salmonella* Enteritidis

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ABSTRACT

Background: Currently, natural food preservation methods are explored, one of which includes the use of herbs and spices.

Methods: The study assessed the effect of herbs and spices; either opened directly before the test or opened and stored for three months; on the survival of *L. monocytogenes* and *S.* Enteritidis bacilli, isolated from meat. Moreover, the microbiological purity of the investigated herbs and spices was evaluated. The research consisted of the analysis of inhibition zone patterns around the wells with spice pulp after the incubation period.

Results: Varied influence of herbs and spices on the survival of bacilli was reported, depending on the species. The strongest impact against *L. monocytogenes*, among freshly opened spices, had: granulated garlic (38.63 mm), whole cloves (28.87 mm), savoury (22.25 mm), ground cinnamon (22.13 mm), ground ginger (18.75 mm). As for *S.* Enteritidis, in the group of freshly opened spices, the strongest effect was found for: granulated garlic (37.25 mm), whole cloves (31.50 mm), and ground cinnamon (18.16 mm). It was reported that the storage of open spices caused a decrease in antimicrobial activity against *L. monocytogenes*, except for cloves, oregano, hot pepper, chilli, sage and turmeric. In the case of *S.* Enteritidis, the following stored spices were not effective: cinnamon, ground black pepper, sage, oregano, basil, tarragon, marjoram, rosemary, coriander, green mint, hot pepper, chilli, curry.

Conclusions: It was confirmed, that herbs and spices, because of its antimicrobial activity can be used, e.g. for food preservation, minimizing the amount of chemical additives applied to the product and extending its shelf-life.

Key words: *L. monocytogenes*, *S.* Enteritidis, spices, herbs, meat, food preservation

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Introduction

Both herbs and spices are an important ingredient of most dishes. They have also application in cosmetology and medicine. Spices include leaves (mint, rosemary), flowers (cloves), bulbs (garlic, onion), fruit (cumin, red chilli), stems (cinnamon) and rhizomes (ginger) of plants. All spices obtained from plants were considered safe for the consumer (GRAS, Generally Recognized As Safe) [1]. It is assumed that over 100 different spices are produced globally. Asia is the leader in its production [2]. Both herbs and spices are a rich source of bioactive chemical compounds, such as vitamins, macro- and micronutrients, glycosides, alkaloids, tannins, flavonoids, phenols, organic acids and saponins. Bioactive substances of spices and herbs can be classified into volatile and non-volatile compounds, the former being mostly responsible for the antimicrobial properties of spices. The volatile active compounds of spices were divided into four groups: terpenes, terpenoids, phenylpropenes and „other", e.g. degradation products [3, 4].

Food poisonings and infections are a serious public health problem, both for consumer and food producers [4]. Current food preservation technologies;

chemical preservatives, heat treatment, packaging in a modified atmosphere or vacuum packaging; are not fully effective in the elimination of certain bacteria, such as *L. monocytogenes* in food products [5]. Meat and dairy products are the most susceptible to microbial contamination [4]. Over 1340 plants with proven antimicrobial activity have been identified, from which over 30,000 compounds have been isolated. It is difficult to determine the sensitivity of microorganisms to herbs and spices. Their effectiveness depends on the pH, temperature and amount of oxygen during storage and the concentration of essential oils and active compounds (conditions of plants growth and harvest) [5]. Active substances can affect the microbial structure; cell membrane or genetic material; and metabolism; e.g. cause disorders in enzymes synthesis and function [3, 6, 7]. Gram-negative bacteria, such as *Escherichia coli* and *S.* Enteritidis, are less susceptible to the action of antimicrobials; the presence of lipopolysaccharide is bounding the diffusion of phenolic compounds; than Gram-positive bacteria (*Staphylococcus aureus*, *L. monocytogenes*, *Bacillus cereus*) due to the direct interaction of their cellular membrane with lipophilic active compounds of herbs and spices [1].

The aim of the study was to assess the impact of commercially available herbs and spices; freshly open or opened and stored for three months at room temperature; on the survival of *L. monocytogenes* and *S.* Enteritidis strains isolated from meat. Moreover, the microbiological purity of herbs and spices used in the study was evaluated.

Materials and methods

Materials

The biological material included eight strains of *L. monocytogenes* and eight strains of *S.* Enteritidis isolated from meat, obtained from the collection of the Department of Microbiology of L. Rydygier Collegium Medicum, UMK in Bydgoszcz.

The study included 20 commercially available herbs and spices (chili, hot pepper, sweet pepper, curry, turmeric, cinnamon, whole cloves, ground black pepper, ground ginger, granulated garlic, green mint, sage, thyme, basil, oregano, savory, marjoram, tarragon, coriander, rosemary) in two variants: freshly open or opened and stored for three months. All spices and herbs used in the study came from a single producer available on the Polish market.

Influence of spices and herbs on the survival of strains tested

In the first step, spices were prepared. For this purpose, 1 g of the seasoning was weighed into a sterile Petri dish. Next, sterile water was added in a volume allowing to obtain the so-called pulp.

The impact of herbs and spices on the microbes survival was evaluated by surface culturing of bacterial suspensions (0.5 on the MacFarland scale) in a buffered saline solution (Avantor) on the Müeller Hinton Agar with 5.0% equine blood and 20 $mg \cdot l^{-1}$ β-NAD (bioMérieux) — *L. monocytogenes*, or the Müeller Hinton Agar (bioMérieux) — *S.* Enteritidis. Next, the tested spice pulp (0.1 g) was applied to the wells drilled with a sterile cork borer. The plates were incubated for 24 h at 37°C. After the incubation period, zones of growth inhibition [mm] were measured around the wells with the spices.

Evaluation of microbiological purity of spices and herbs

The microbiological purity of all 20 spices and herbs tested (opened immediately before the study, and opened and stored for three months at room temperature) was evaluated in accordance with the Polish norm PN-A-86967 [8]. After the incubation period, the species identification of the grown colonies was performed using the MALDI TOF MS apparatus (Bruker) in accordance with the manufacturer's instruction.

Statistical analysis

The statistical analysis was performed using Statistica 12.0 PL (StatSoft) software. To evaluate the differences in microbiocidal activity, depending on the type of spices and the time that has elapsed since their opening, the *post*-hoc Tukey test was used. The differences were considered significant at the probability level $p < 0.05$.

Results

It was found that all spices and herbs opened immediately before the study demonstrated activity against *L. monocytogenes* (Fig. 1). It was also reported that granulated garlic (37.25 mm), whole cloves (31.50 mm), ground cinnamon (18.16 mm), ground ginger (15.88 mm), and hot pepper (11.38 mm) had the strongest antimicrobial activity against *S.* Enteritidis in the group of freshly opened spices (Fig. 2). As for *L. monocytogenes*, the strongest effect among the freshly open spices had: granulated garlic (38.63 mm), whole cloves (28.87 mm), savoury (22.25 mm), ground cinnamon (22.13 mm), and ground ginger (18.75 mm) (Fig. 1). In the group of spices opened immediately before the study, significant differences were found for granulated garlic (Fig. 1). As for basil opened immediately prior to the study, activity against one strain of *L. monocytogenes* was found (Fig. 1). Among the freshly

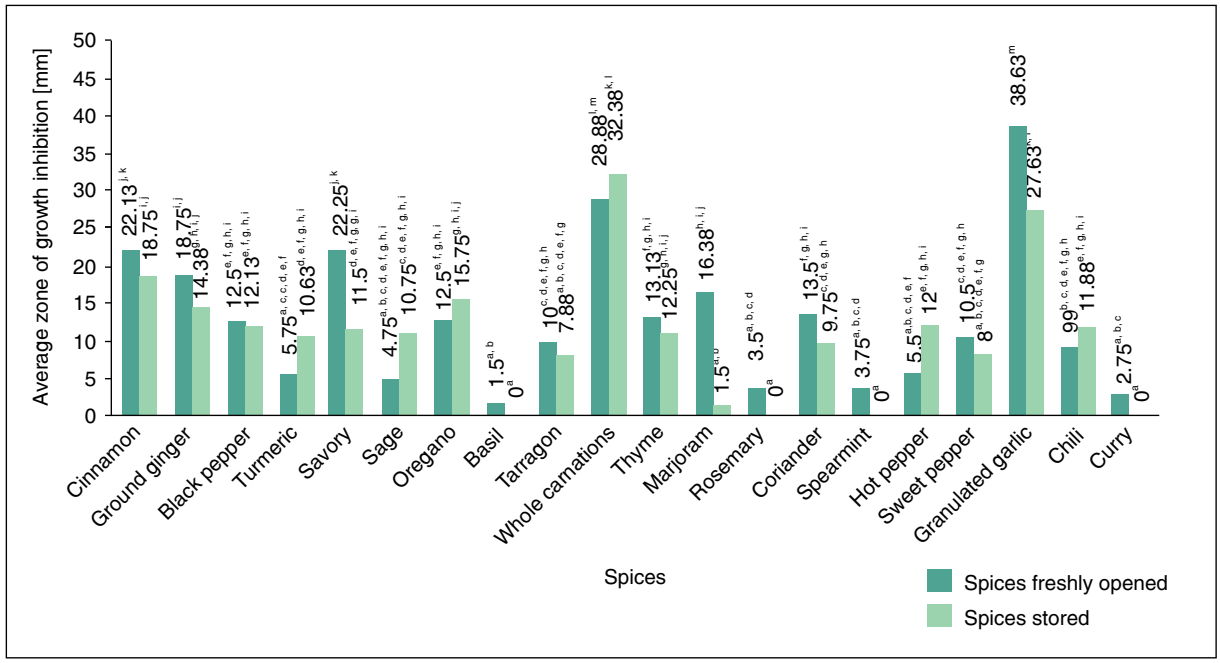

Figure 1. Mean zones of *L. monocytogenes* growth inhibition obtained with spices opened immediately before the test, and opened and stored for three months at room temperature (a, b, c, ... — means marked with different letters differ significantly)

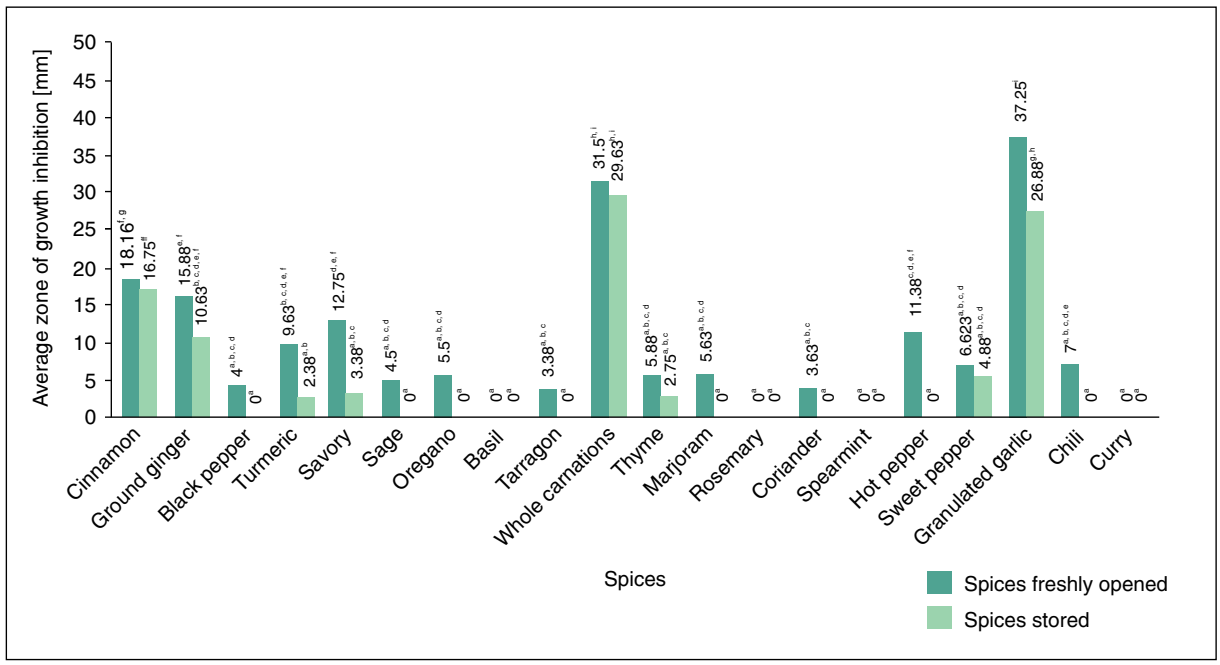

Figure 2. Mean zones of *S.* Enteritidis growth inhibition obtained with spices opened immediately before the test, and opened and stored for three months at room temperature (a, b, c, ... — means marked with different letters differ significantly)

opened basil, rosemary and curry spices, there were no zones of *S.* Enteritidis growth inhibition detected (Fig. 2).

In the present study, no influence of the stored curry, rosemary, basil and green mint spices on *L. monocytogenes* bacilli was reported (Fig. 1). In the group of stored spices, the highest activity against *L. monocytogenes* was found for: whole cloves (33.38 mm), granulated garlic (27.63 mm), ground cinnamon

(18.75 mm), oregano (15.75 mm), and ground ginger (14.38 mm) (Fig. 1). As for stored marjoram, activity against one *L. monocytogenes* strain was observed (Fig. 1). In the present study, in the group of stored spices, the activity against *S.* Enteritidis was demonstrated with whole cloves (29.63 mm), granulated garlic (26.88 mm), ground cinnamon (16.75 mm), ground ginger (10.63 mm), sweet pepper (4.88 mm)), savoury (3.38 mm), thyme (2.75 mm), and turmeric (2.38 mm) (Fig. 2). Significant differences in the spices tested three months after opening were found for cinnamon, ground black pepper, sage, oregano, basil, tarragon, marjoram, rosemary, coriander, green mint, hot pepper, chilli, and curry, which did not show antimicrobial activity against *S.* Enteritidis (Fig. 2).

Larger zones of *L. monocytogenes* growth inhibition were observed in the case of cloves (32.38 mm), oregano (15.75 mm), hot peppers (12.00 mm), chilli (11.88 mm), sage 10.75 mm), and curcuma (10.63) spices tested three months after opening as compared to the spices opened immediately before the study (Fig. 1). As for *S.* Enteritidis, no differences in the antimicrobial activity were found between freshly opened and stored spices (Fig. 2).

The results of the microbiological purity evaluation of the herbs and spices used are shown in Table 1. A significant level of spices contamination was demonstrated, mostly with *Bacillus* species. The number of isolated fungi and molds was within the limits permitted by the Polish standard PN-A-86967: 1998/Az1: 2002 [8].

Discussion

Herbs and spices have been used for culinary purposes for centuries, but also in cosmetology and medicine. The antimicrobial properties of herbs are related to the presence of a number of chemical compounds [4].

In the own study, it was shown that granulated garlic suppressed the growth of *L. monocytogenes* and *S*. Enteritidis most strongly. Scientific research provides a lot of data on a wide range of garlic activity, which affects the survival of both Gram-positive and Gram-negative bacteria, including *Escherichia*, *Salmonella*, *Staphylococcus*, *Streptococcus*, *Klebsiella*, *Proteus*, *Bacillus* and *Clostridium* species (except *C. botulinum*) [4]. The own study also showed that ground cinnamon and whole cloves (both freshly opened and stored after opening) inhibited the growth of the tested strains. The direct antimicrobial activity of ground cinnamon and cloves against *E. coli*, *S. aureus*, *Brochothrix thermosphacta, Lactobacillus rhamnosus* and *Pseudomonas fluorescens* isolated from meat was presented by Kuanga et al. [9]. They showed that ground clove powder had a strong inhibitory effect on the five microorganisms tested (MIC: 1.0% w/v — 2.0% w/v) [9]. On the other hand, Bayoub et al. [10] reported that clove extract was the most effective inhibitor of *L. monocytogenes* as compared to the other 12 plant extracts based on ethanol (MIC: 0.24 mg/ml). In the own study, no influence of basil, curry rosemary (freshly opened spices) against *S*. Enteritidis. However, Weerakkody et al. [11] found that hexane rosemary extract showed a higher antibacterial activity than aqueous extracts in relation to all bacteria tested, except *S.* Typhimurium (MIC: 1.25–5.0 mg/ml). Weerakkody et al. [11] also reported high activity of lemongrass, mountain pepper and rosemary against *S. aureus*, *E. coli*, *S.* Typhimurium and *L. monocytogenes*. Moreover, Sandasi et al. [12] found that basil, oregano, thyme, rosemary and ginger limited the mobility of *E. coli* 0157: H7 and *P. aeruginosa*, while thyme and rosemary reduced the adhesion of *L. monocytogenes* cells to PVC (polyvinyl chloride), and thus, limited the biofilm development.

In the present study, no effect of curry, rosemary, basil and green mint (spices stored) on *L. monocytogenes* was found. Antibacterial activity of essential oils from oregano and thyme against *L. monocytogenes* and *E. coli* O157: H7 strains was demonstrated by Aboaba et al. [13] and Govaris et al. [14]. Also, Dimitrijevic et al. [15] demonstrated the effect of essential oils of thyme, rosemary and oregano against *L. monocytogenes*. Among the stored spices activities against *S*. Enteritidis was not shown for cinnamon, ground black pepper, sage, oregano, basil, tarragon, marjoram, rosemary, coriander, green mint, hot pepper, chilli and curry. On the other hand, Bayou et al. [10] found that cloves extract was the most effective inhibitor of *L. monocytogenes* as compared to the other 12 plant extracts based on ethanol (MIC: 0.24 mg/ml). Babacan et al. [16] evaluated the antimicrobial effect of oregano extract on several *Salmonella* serotypes. Growth zones of 15, 19 and 16 mm-in-size were reported for *S.* Gallinarum, *S.* Enteritidis and *S.* Typhimurium, respectively [16]. However, de Medeiros Barbosa et al. [17] demonstrated the antibacterial effect of essential oils from oregano and rosemary against *E. coli*, *L. monocytogenes* and *Salmonella spp*. Al-Turki [18] described the antimicrobial action of hydrosols from five spice (thyme, peppermint, sage, black pepper and garlic) against *B. subtilis* and *S.* Enteritidis. The Author stated that garlic provided a stronger antibacterial effect against *B. subtilis* and *S.* Enteritidis in comparison with thyme, mint, sage and black pepper [18].

The assessment of microbiological purity of the tested spices, available on the Polish market, showed their significant contamination. Nevertheless, the results were within the limits allowed by the Polish Norm. Brużewicz and Malicki [19] evaluated the microbiological purity of herbs and spices on the Polish market. They found

Table 1. Results of the microbiological purity evaluation of the spices investigated

Spice	Spices tested immediately after package opening			Spices tested three months after package opening		
	TSA agar	MacConkey agar	Sabouraud agar	TSA agar	MacConkey agar	Sabouraud agar
chili	+ (1 colony)	–	+ (1 colony)	+ *Staphylococcus camaieu*	–	–
hot pepper	+ (uc*) *Bacillus pumilus*	–	–	+ *Bacillus pumilus, Micrococcus luteus*	–	–
sweet pepper	–	–	–	–	–	+ (1 colony)
curry	+ (confluent growth) *Bacillus pumilus*	–	+ (1 colony)	+ (approx. 1000 colonies) *Paenibacillus viridis, Bacillus clausii*	–	–
turmeric	+ (1 colony)	–	–	+ *Staphylococcus warneri*	–	–
cinnamon	–	–	–	+ (3 colonies)	–	–
whole cloves	+ (uc)	–	–	+ (confluent growth)	+ (1 colony)	+ (1 colony)
ground black pepper	+ (confluent growth)	–	–	+ (confluent growth)	–	–
ground ginger	+ (confluent growth, *Bacillus pumilus*) + (*Lysinibacillus fusimoris*)	+ (2 colonies)	+ (3 colonies)	+ (confluent growth) *Bacillus pumilus*	–	–
granulated garlic	+ (uc)	+ (1 colony)	–	+ (confluent growth) *Bacillus pumilus*	+ (15 colonies) *Escherichia vulneris*	–
spearmint	–	–	–	+ (3 colonies)	–	–
sage	+ (uc)	–	+ (4 colonies)	+ (confluent growth) *Bacillus pumilus*	+ (2 colonies) *Pantoea calida*	+ (5 colonies)
thyme	+ (uc) *Bacillus licheniformis*	–	–	+ (20 colonies) *Bacillus licheniformis, Bacillus pumilus*	–	+ (1 colony)
basil	+ (uc)	–	–	–	–	–
oregano	+ (uc) *Bacillus pumilus*	–	+ (4 colonies)	–	–	–
savory	+ (6 colonies) *Bacillus licheniformis*	–	–	+ (1 colony) *Bacillus licheniformis*	–	–
marjoram	+ (uc) *Bacillus licheniformis*			+ (confluent growth)	–	–
tarragon	+ (uc) *Bacillus pumilus*	–	–	+ (approx. 600 colonies) *Bacillus simplex*	–	–
coriander	+ (uc) *Bacillus pumilus*	–	–	+ (12 colonies) *Bacillus pumilus, Bacillus simplex*	–	+ (4 colonies)
rosemary	+ (uc) *Bacillus pumilus*	+ (uc) *Pantoea agglomerans*	+ (5 colonies)	–	–	–

that the level of contamination with oxygen microflora and saprophytic fungi oscillated within the limits allowed by the Polish standard, at 10^5 CFU × g^{-1} and 10^3 CFU × g^{-1}. The presence of *E. coli* and *Salmonella*, as well as coagulase-positive staphylococci, was detected in none of the samples tested. Based on the obtained data; related to the changes in the number of microorganisms noted during the storage period; the following product contamination categories were distinguished: 1) remained unchanged (pepper, nutmeg, basil), 2) decreased (garlic, onion, coriander, parsley, paprika, turmeric) or 3) increased (tomato) [19]. Lins [20] found that *Salmonella* spp. and coagulase-positive staphylococci were not detected in 25 g of the spices tested.

Conclusions

The antimicrobial activity of herbs and spices against *L. monocytogenes* and *S.* Enteritidis demonstrated in this study can be used in the food industry, e.g. for food preservation, minimizing the amount of chemical additives applied to the product and extending its shelf-life. Attention should be paid to the durability of herbs (active substances) during storage and the microbiological contamination of commercially available spices. It is important to broaden the research on the characteristics of active compounds in spices and herbs, and the mechanism of their action on microorganisms.

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VIA MEDICA

ORIGINAL ARTICLE

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Extracellular divalent ions modulate TREK-2-like channel conductance in prefrontal pyramidal neurons in rats

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ABSTRACT

Background: The aim of the study was to investigate, with the use of the patch clamp technique, the dependence of the conductance of the TREK-2-like potassium leak channel in the medial prefrontal cortex pyramidal neurons on the presence of extracellular magnesium and calcium ions. It is suspected that TREK-2 channels regulate mood and may be associated with the pathophysiology of depression. Since magnesium and calcium deficiency contribute to depressive symptoms, we investigated how TREK-2- like channel pore properties change in the absence of divalent cations.

Results: Single-channel currents were recorded in a cell-attached configuration in enzymatically dispersed pyramidal neurons of the prefrontal cortex in rats. Spontaneous TREK-2-like channel activity was recorded either in the presence or absence of magnesium and calcium ions in extracellular solution. A significant increase in the inward channel conductance was observed when divalent cations were removed from the extracellular solution. Inward rectification was also increased when the bath temperature was raised to 34-37°C.

Conclusions: The study confirmed that the activity of TREK-2-like channels is affected by the presence of magnesium and calcium ions in the extracellular solution. Therefore, *in vivo,* the TREK-2-like channel may possibly participate in the prefrontal cortex dysfunction associated with the deficiency of divalent cations.

Key words: magnesium and calcium homeostasis; depression; TREK-2 channels; leak potassium channels; patch-clamp

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Introduction

Dysfunction of the prefrontal cortex occurs in many neuropsychiatric diseases. Some of these, such as depression, schizophrenia, mania, or anxiety disorders, may accompany magnesium and calcium homeostasis dysregulation [1–5]. Neuronal excitability is controlled by the activity of ion channels. Therefore, understanding the impact extracellular ions have on the kinetics of ion channels in the prefrontal cortex pyramidal neurons may be important.

TREK-2 is a member of the two-pore domain channels that form a family of the leak (or background) K^+ channels. The main function of leak potassium channels is to stabilize the resting membrane potential, which affects the excitability of neurons. It was recently reported that TREK-2-like channels are present in pyramidal cells in the prefrontal cortex of rats [6]. Thus, the excitability of prefrontal pyramidal cells can depend on the activity of TREK-2-like channels, not only in physiological but also in pathophysiological conditions.

Although the TREK-2 channels are distinct from voltage-activated channels (Kv family) or inward rectifier channels (Kir family), to a certain degree their kinetics resemble those of Kv and Kir channels. The probability of opening (Po) of TREK-2 channels increases with membrane depolarization [6], and the inward conductance exceeds the outward conductance (the latter does not result from asymmetrical ion concentration) [7]. In our work, we examined how the rectifying properties of the TREK-2-like channel depend on the presence of divalent ions, such as Mg^{2+} and Ca^{2+}, in extracellular solution in temperature close to physiological.

Because it is believed that TREK channels family may be involved in the pathogenesis of depression [8–10], understanding the influence of Mg^{2+} and Ca^{2+} ions on the activity of TREK-2 channels may contribute to the understanding of the mechanisms

leading to depression in hypomagnesaemia and hypocalcaemia.

Methods

The experimental procedures used in this study conform to institutional and international guidelines for the ethical use of animals. The experiments were performed on young (20-days-old) male Wistar rats. Preparation of the slices and the procedure of enzymatic tissue dissociation were performed as described previously [6]. Voltage-clamp recordings were performed in the cell-attached configuration using an Axopatch 1D amplifier (Axon Instruments) and pClamp 9.0 software. Data were filtered at 5 kHz, digitized at 50 kHz and stored on a computer.

The cells were perfused with bath solution containing (in mM): KCl (145), $CaCl_2$ (2), $MgCl_2$ (2), glucose (10), $LaCl_3$ (0.0015) and HEPES (10). The pH of the solution was adjusted to 7.4 using NMDG, and the osmolality was adjusted to 330 mOsm using sucrose. The pipette solution contained (in mM): potassium acetate (130), HEPES (10), $MgCl_2$ (2), $CaCl_2$ (2), TTX (0.005), and $LaCl_3$ (0.0005). The pH of the solution was 7.4 and was adjusted using NMDG; its osmolality was 280–300 mOsm. In experiments with Mg^{2+}-free solution, the composition of the pipette solution was as follows: potassium acetate (122), HEPES (10), $CaCl_2$ (0.1), EGTA (0.1), KCl (8) and TTX (0.005), and $LaCl_3$ (0.0005).

The unitary current amplitudes were determined from all-point amplitude histograms fitted with Gaussian functions. Only full openings, i.e. openings with maximum amplitude, were included in the current analysis.

To evaluate statistical significance between four groups, we used one-way *ANOVA.* If not stated otherwise, p values refer to Tukey's post-hoc tests.

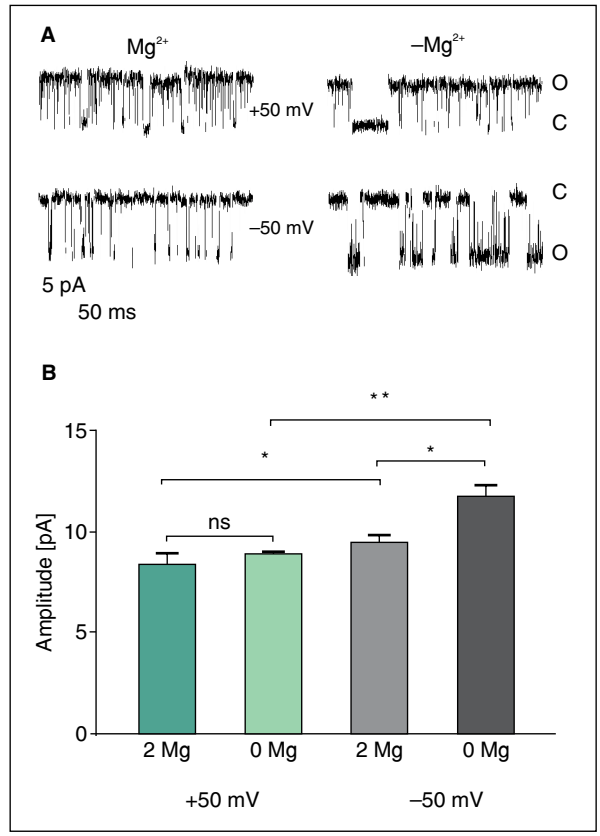

Figure 1. Effect of extracellular Mg^{2+} on potassium TREK-2 like currents.
A) Single channel recordings of TREK-2 like currents with 2 mM or without Mg^{2+} in the pipette solution at two membrane potentials. Positive current represents outward potassium current, negative — inward current; c — closed channel, o — open channel; high pass filters set at 5 kHz. **B)** The mean unitary current (absolute value) measured at depolarization (+50 mV) and hyperpolarization (-50 mV) of cell membrane with 2 mM or without Mg^{2+} in the pipette solution; *p < 0.02, **p < 0.01, ns p > 0.88.

Results

In the study presented here, we recorded single-channel potassium currents in the pyramidal prefrontal cortex of rats. Recordings were carried out in high extracellular potassium concentration in cell-attached configuration. In the study, we investigated the effect of divalent ions and temperature on the amplitude of TREK-2-like currents that appeared spontaneously in membrane depolarization and hyperpolarization. Identification of the channel as a TREK-2-like channel in its non-canonical isoform (b or c) has been described previously [6] and was performed on the basis of pharmacological and biophysical properties and with the use of immunofluorescence together with confocal microscopy. Non-canonical isoforms result from alternative splicing [7].

Channel rectifying properties in the presence (2 mM Mg^{2+} and 2 mM Ca^{2+}) and the absence of divalent ions (0 Mg^{2+}, 0.1 mM Ca^{2+}, 0.1 mM EGTA, a trace quantity of calcium ions were present) at room temperature are shown in Fig.1. A voltage of +50 mV (depolarization) or -50 mV (hyperpolarization) was applied to cell membranes and single-channel currents were recorded at room temperature. In both variants, the amplitude of the inward current evoked by negative potential was higher that of the outward current. Single channel conductance values *S* were as follows (subscribe D — depolarization, H — hyperpolarization, MgCa — presence of divalent ions, EGTA — 0 mM Mg^{2+}):

$$S_{D,MgCa} = 167.2 \text{ pS} \pm 10.5 \text{ pS} \ (n = 7)$$
$$S_{H,MgCa} = 188.1 \text{ pS} \pm 8.2 \text{ pS} \ (n = 7),$$
$$S_{D,EGTA} = 177.2 \text{ pS} \pm 1.9 \text{ pS} \ (n = 4),$$
$$S_{H,EGTA} = 234.4 \text{ pS} \pm 1.1 \text{ pS} \ (n = 4).$$

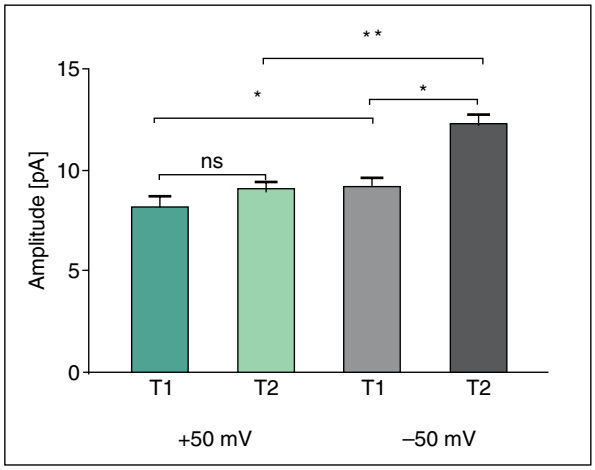

Figure 2. Effect of temperature on potassium TREK-2 like currents in the presence of 2 mM Mg^{2+} in the pipette solution. T1 21–25°C, T2 34–37°C. *p < 0.02, **p < 0.004, ns p > 0.036

The obtained results indicate that the studied channel had rectifying properties, i.e. outward conductance $S_{D\,MgCa}$ was significantly lower than inward conductance $S_{H,MgCa}$ (p = 0.0037) and that properties of inward conductance S_H were dependent on presence of divalent cations ($S_{H,MgCa}$ vs $S_{H,EGTA}$, p = 0.008; $S_{D,\mathrm{MgCa}}$ vs $S_{D,EGTA}$, non-significant).

Next, we measured the conductance of the channel at room temperature T1 (~25°C) and at higher temperature T2 (temperature 34°–37°C) in the presence of divalent ions. Unfortunately, the instability of the seal at higher temperature in the absence of divalent cations made recording quality under these conditions insufficient for analysis. Single-channel conductances S were as follows (subscribe D — depolarization, H — hyperpolarization, T1 — room temperature, T2 — high temperature):

$$S_{D,T1} = 167.2\ \mathrm{pS} \pm 10.5\ \mathrm{pS}\ (n = 7),$$
$$S_{H,T1} = 188.1\ \mathrm{pS} \pm 8.2\ \mathrm{pS}\ (n = 7),$$
$$S_{D,T2} = 186.4\ \mathrm{pS} \pm 8.3\ \mathrm{pS}\ (n = 7),$$
$$S_{H,T2} = 250.5\ \mathrm{pS} \pm 10.6\ \mathrm{pS}\ (n = 7).$$

The conductances marked with the symbols $S_{D,T1}$ and $S_{H,T1}$ are the same as conductances $S_{D,MgCa}$ and $S_{H,MgCa}$ respectively. The results indicate that the increase in temperature from T1 to T2 increased the inward conductance by 62 pS ($S_{H,T1}$ vs. $S_{H,T2}$, p < 0.006), while the increase of the outward conductance was statistically insignificant ($S_{D,T1}$ vs. $S_{D,T2}$, p = 0.36). This means that the temperature has a significant influence on the inward conductance (Fig. 2).

Discussion

In the presented work, we measured single-channel potassium currents with a large conductance (> 150 pS) in rat pyramidal prefrontal cortex neurons. These currents have been previously identified as conducted by TREK-2-like channels in its non-canonical form b or c [6]. We performed experiments with two different solutions in a pipette (pipette solution corresponds to the extracellular environment). One solution contained divalent ions at a concentration of 2mM (2mM Mg^{2+}, 2mM Ca^{2+}), the other solution was Mg^{2+} -free and contained a trace concentration of Ca^{2+} ions (0.1 mM Ca, 0.1 mM EGTA).

Our most important result is the confirmation of the effect of extracellular divalent ions on the inward conductance of the recorded channels and presenting that the inward rectification depends on temperature: the difference between outward and inward conductance is bigger in higher temperature (Fig. 1). The effect of removing extracellular Mg^{2+} is similar to the effect of increasing temperature. In the absence of divalent ions in the extracellular solution, the amplitude of the inward current and thus the inward conductance of the channel increased (Fig. 1). The influence of extracellular magnesium ions on channel conductance can be analogous to the blocking mechanism of Kir channels by intracellular magnesium ions [11, 12]. Most likely, magnesium ions from the extracellular side penetrate into the entrance region of the channel pore and block the potassium current. That penetration can be impaired at higher temperature. Publications indicate that extracellular Ca^{2+} does not usually block potassium channels. An exception is the HERG channel [13]. The mechanism of action of divalent ions was not, however, the subject of the work presented here.

Our research suggests that the level of divalent ions (Mg^{2+}, Ca^{2+}) in extracellular solution may have a significant effect on the functioning of TREK-2-like potassium channels *in vivo*. Because these channels are expressed in the prefrontal pyramidal cells, the TREK-2-like channels possibly affect the functions of the prefrontal cortex in a manner dependent on the concentration of Mg^{2+} or Mg^{2+} and Ca^{2+}.

There are studies suggesting that the activity of TREK-1 and TREK-2 ion channels can affect mood regulation and showing that selective serotonin reuptake inhibitors affect the activity of TREK-1 and TREK-2 channels [8–10,14–16]. These channels are therefore a promising goal of drug action in new therapies for the treatment of depressive disorders [17].

A variety of neuromuscular and psychiatric symptoms, including depression, was reported in dysregulation of magnesium homeostasis [2, 3, 5]. Although the antidepressant activity of magnesium is mostly ascribed to the activity of the NMDA receptor, the mechanism of the antidepressant effect of magnesium is not yet fully understood [2]. Our work suggests that in the conditions of disturbed homeostasis of divalent ions, TREK-2 channels can interfere with the functioning of the cortex

pyramidal cells. Therefore, it would be important to examine the role of TREK-2 channels in depression accompanying hypomagnesemia in future studies.

Conclusions

The activity of TREK-2-like channels is affected by the presence of magnesium and calcium ions in the extracellular solution. *In vivo* the TREK-2-like channel may possibly participate in dysfunctions associated with the deficiency of divalent cations like depression, schizophrenia, anxiety.

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VIA MEDICA

ORIGINAL ARTICLE

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TTX-resistant sodium currents in medial prefrontal cortex pyramidal neurons depend on extracellular Ca^{2+} concentration

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ABSTRACT

Background: Previous reports reported the presence of TTX-resistant Nav1.8 and Nav1.9 sodium channels in the cortex pyramidal neurons. A characteristic feature of Nav1.9 channels is activation at voltages close to — 70 mV. Therefore, they do not participate directly in the action potential but contribute to the regulation of the resting membrane potential. Their physiological role is modulation of cell excitability. The aim of the study was to investigate, with the use of patch clamp technique, the dependence of the activation thresholds of TTX–resistant sodium currents on the concentration of extracellular calcium in medial prefrontal cortex pyramidal neurons in rats.

Results: The recorded values of the threshold of the voltage-dependent sodium currents were in the range of -65 mV to -75 mV. This suggests that the sodium currents may result from the presence of Nav1.9 channels in the rat pyramidal neurons. The threshold for the activation of sodium currents depended on the concentration of Ca^{2+}. Increasing the concentration of calcium ions in the extracellular solution by 5 mM caused the depolarizing shift in the activation potential by about 10 mV. The effect of calcium ion concentration on the potential of TTX-resistant currents activation suggests that Nav1.9 channels are modulated by extracellular Ca^{2+} concentration.

Conclusions: The study has experimentally confirmed that TTX-resistant channels are present in the cell membrane of the rat prefrontal cortex pyramidal neurons and may, therefore, take a physiological role in the conductivity of sodium currents in a manner dependent on the concentration of extracellular ions.

Keywords: sodium currents TTX-resistant, Nav1.9 ion channel, pyramidal neurons, patch-clamp

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INTRODUCTION

Sodium channels present in cell membranes enable sodium ions to flow into the cytoplasm, thus increasing the electric potential of the membrane or depolarization. This phenomenon is a key element of many physiological processes, one of the most important of which is generation and transmission of neural impulses, including those related to pain. Sodium channels comprise a varied group, with individual members having separate coding genes, their specific opening and closing kinetics, as well as location and physiological functions.

The voltage-gated $Na_v1.9$ sodium channel is formed by an α subunit coded by the SCN11A gene. It is preferentially expressed in neurons of dorsal root ganglia (DRG), trigeminal ganglion neurons and in the enteric nervous system [1, 2]. The relation between $Na_v1.9$ channels and pain is confirmed by cases of people with mutations of this channel, suffering from neuropathic pain [3, 4]. Mutations of this channel caused depolarization of resting potential of neurons, facilitating spontaneous generation of action potentials. On the other hand, mutations of this channel were also found in people who did not perceive pain e.g. upon fractures or injuries [5]. $Na_v1.9$ channels are activated at the potential of approximately -70 mV, which is lower than activation potential for other voltage-gated sodium channels, are inactivated slowly, i.e. the sodium current disappears slowly over the duration of the activating stimulus and is resistant to TTX (tetrodotoxin), a blocker of most voltage-gated sodium channels. Because of this characteristic, it is thought that $Na_v1.9$ channels

regulate the excitability of pain receptors and are an important element in the transmission of pain signals to the central nervous system [1, 6].

It was accepted until recently that $Na_v1.9$ channels are absent in the central nervous system [7], however, the presence of the $Na_v1.9$ protein was proven in pyramidal neurons of the V layer of prefrontal cortex in rats using confocal microscopy and $Na_v1.9$ antibody labelling [8, 9]. Previous studies showed that TTX-resistant sodium current in pyramidal neurons of rat cortex displays characteristics typical for Nav1.8 and Nav1.9 channels [9, 10]. Thus, depending on the patch clamp protocol used, it may be a result of currents from both these channels. The Nav1.9 channel probably impacts the membrane potential of pyramidal cells because of the fact that its activation potential is close to the resting potential of the cell [8]. The goal of this work was to study the influence of extracellular calcium on the activation threshold of TTX-resistant sodium currents in such cells using the patch clamp technique. This means that the study of calcium influence on TTX-resistant currents, a component of which includes currents conducted by the Nav1.9 channel is a study on the mechanism in which calcium regulates the excitability of pyramidal cells.

Under physiological conditions, the concentration of calcium in blood varies in the range between 2.25 mM and 2.75 mM. Approximately half of the calcium exists in a free ionized form. The influence of chances to calcium concentration on the excitability of the nervous system is proven by symptoms related to hypo- and hypercalcemia. There are many typical, neurological symptoms of hypocalcemia. Typical symptoms include tingling, tetany and muscle contractions. Severe hypocalcemia may cause seizures [11–16] or be responsible for the paradoxical reaction (seizure intensification) to phenytoin administered as an anti-seizure medication [17]. Neurological and neuromuscular symptoms of hypercalcemia yield a different clinical picture and include changes such as drowsiness, coma, decreased muscle strength, adynamia or periodic muscle paralysis [18]. The listed symptoms indicate that hypocalcemia is the cause of symptoms related to neuromuscular hyperexcitability, while hypercalcemia is the cause of decreased neuronal or neuromuscular excitability. Mechanisms responsible for increased excitability of the nervous system in hypocalcemia or for decreased excitability in hypercalcemia are not fully known [19].

Studies performed on brain slices indicate that a decrease of calcium concentration in the extracellular solution stimulates spontaneous neural activity. It was shown that pre-incubation of samples in a solution with low calcium concentration in the presence of synaptic transmission inhibitors results in spontaneous nonsynaptic epileptiform activity, increased resting potential of the cells and a decrease of the action potential activation threshold [20–22]. On the contrary, pre-incubation of brain slices in a solution with increased calcium concentration inhibits spontaneous neural activity caused by the increased K^+ concentration [23]. These observations indicate that calcium regulates the activity of ion channels engaged in the determination of resting potential and of channels responsible for action potential generation. In this work, we have studied if and how an increase of extracellular calcium concentration influences the activation potential of TTX-resistant currents. The study was performed on freshly isolated and enzymatically/mechanically dispersed pyramidal neurons of the rat medial prefrontal cortex [24].

Material and methods

The study was performed on slices of the medial prefrontal cortex of 3 weeks old male Wistar rats. After application of anaesthesia using ethyl chloride and decapitation, the brain was placed in liquid cooled down to 0°C, with the following composition (mM): saccharose 234, HEPES 15, glucose 11, $MgSO_4$ 4, KCl 2.5, ascorbic acid 1, NaH_2PO_4 1. The part of the brain containing frontal lobes was cut into 300 μm-thick slices in a vibratome and placed in a liquid kept at room temperature, with the following composition (mM): NaCl 118, $NaHCO_3$ 25, glucose 6, $MgSO_4$ 3, KCl 2.5, $NaHPO_4$ 1,25, $CaCl_2$ 0,5, glutathione 0.01, saturated with carbogen (95% O_2, 5% CO_2). The slices were incubated in this solution for 1–6 hours. In order to isolate individual pyramidal neurons, the slices were placed in a solution with the following composition (mM): sodium isothioate 140, glucose 20, HEPES 15, $MgCl_2$ 4, KCl 2, $CaCl_2$, a part of the prefrontal cortex was cut out and subjected to enzymatic activity in the same liquid containing added protease (20 μg/20 ml) for 20 minutes at 33–37°C. Parts of the prefrontal cortex were mechanically dispersed using a glass pipette. The cell suspension was placed in a patch clamp recording chamber, on a glass pre-coated with Poly-L-lysine (Sigma-Aldrich). Electrophysiological tests were performed after approximately 5 minutes.

The functional study of TTX-resistant channels used the patch clamp technique in the whole-cell configuration, which enables recording of currents from the whole cell. The research setup included an Axopatch 1D amplifier, a digital-analog converter Digidata 1440A, a computer and a reverse microscope Olympus IX71. The pClamp10 program (Molecular Devices, USA) was used to record and analyse the data. A silver chloride measurement electrode was placed inside a pipette containing solution with the following composition (mM): CsF 110, CsCl 20, NaCl

10, HEPES 10, $MgCl_2$ 2, EGTA 2. The extracellular solution included (mM): NaCl 130, TEA-Cl 30, HEPES 10, $MgCl_2$ 2, $CaCl_2$1,5, 4AP 1, $CdCl_2$ 0.1. This solution was replaced in individual experiments with an identical liquid containing TTX (0.5 μM) or:

A) a solution with low sodium ion concentration, in which the control 130 mM NaCl was replaced with a mixture of 10 mM NaCl and 120 mM choline,

B) an extracellular solution with increased Ca^{2+} concentration of 6.5 mM and $CdCl_2$ concentration of 0.2 mM. All solutions, in which currents were recorded contained $CdCl_2$ and, if TTX-resistant currents were recorded, also 0.5 μM TTX. Cadmium was added in order to block calcium channels.

Once a high resistance connection was established between the measurement pipette and the cell membrane („giga seal"), the membrane was ruptured and if the resistance of the connection between the pipette and the cell and pipette resistance were satisfactory (> 300 MΩ and < 10 MΩ, respectively), cellular currents were recorded. In order to record the current, a linearly increased potential in the range between -110 mV and 80 mV and with the slope of 0.25 mV/ms was applied to the cell membrane. The potential value between recordings was maintained at -70 mV. A brief (500 ms) potential impulse of -110mV was applied before the recording in order to remove the inactivation of sodium channels. All measurements were performed at room temperature.

Before the analysis of TTX-resistant currents, the linear component corresponding to leak current was subtracted from the recorded current, Fig. 1.

The statistical analysis employed:

C) the Shapiro-Wilk test and the Kolmogorov–Smirnov test in order to verify the hypothesis of conformity of the distribution of the studied characteristics with a normal distribution;

D) the *t*-Student test for dependent samples in order to verify the hypothesis of statistically significant differences between the I_{max} and V_{act} values recorded under different experimental conditions;

E) Wilcoxon signed-rank test, in order to verify the hypothesis of significant differences between the recorded values, if the distribution of the tested characteristic did not conform to a normal distribution;

The analysis was performed on current recordings performed on 13 pyramidal neurons obtained from 5 rats.

Results

The tested cells displayed intracellularly oriented currents, some of which were resistant to TTX (Fig. 2A).

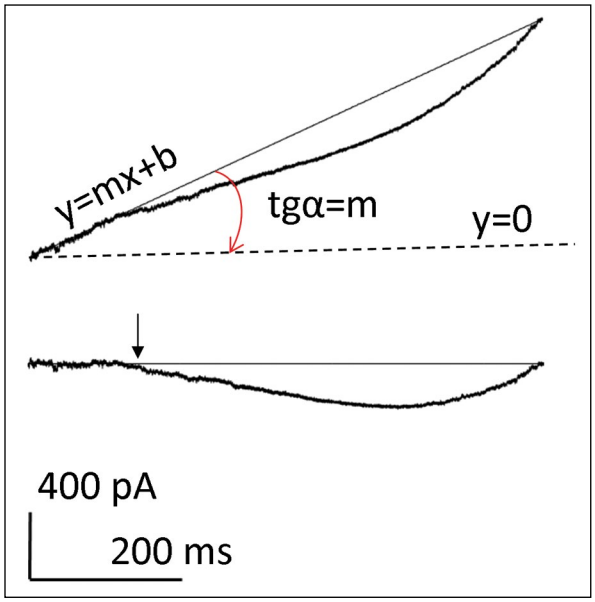

Figure 1. Voltage ramp (from –110 mV to 60 mV) was applied to evoke sodium currents. The leak currents were fitted by a line to the portion of the current that was more negative than -80 mV and then subtracted. The arrow indicates the threshold of the voltage-dependent component of the inward current.

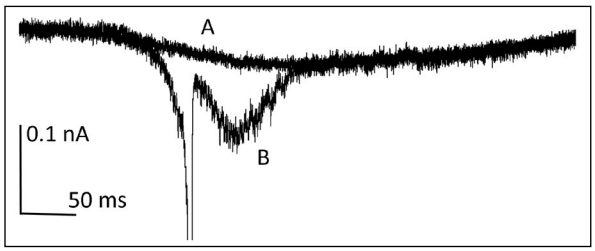

Figure 2. The effect of TTX on the sodium currents. Voltage ramp: from -75 mV (left) to 30 mV(right). A — 0.5 μM TTX, B — no TTX added.

If TTX was absent in the extracellular solution, the recordings showed quickly activated and inactivated sodium currents characteristic for action potentials and slowly activated and inactivated currents susceptible to TTX (Fig. 2B).

TTX-resistant currents depended on the concentration of sodium ions (Fig. 3). The extracellular solution with low Na^+ concentration (choline was the main cation in the solution) showed an amplitude of the peak TTX-resistant current (I_{max}) lower than in the control solution (30.8 ± 10.5 pA vs. 87.7 ± 15.5 pA, respectively, $n = 13$, $p < 0.05$). The activation potential of the TTX-resistant current (V_{act}) was -74.8 ± 2.0 mV in the control solution.

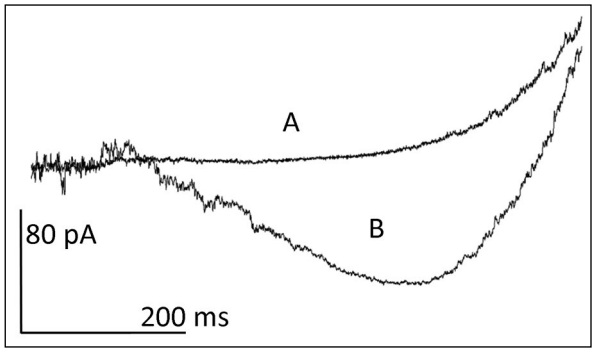

Figure 3. The effect of reducing the sodium concentration on the recorded currents. Voltage ramp: from -110 mV (left) to 60 mV(right). **A** — 10 mM Na+; **B** — 130 mM Na+. TTX (0,5 μM) was present in A and B.

The TTX-resistant solution was characterised by slow activation and slow inactivation.

An increase of the extracellular Ca^{2+} concentration from 1.5 to 6.5 mM did not result in statistically significant differences in the values of the peak current I_{max} (63.2 ± 23.5 pA vs. 96.0 ± 51.9 pA, $n = 6$, $p > 0.5$), but it influenced the activation potential significantly. The activation potential was: -69.0 ± 3.5 mV for the control sample and -59.0 ± 3.4 mV for 6.5 mM Ca^{2+}. An increase of the Ca^{2+} concentration by 5 mM resulted in a statistically significant (*t*-Student test for dependent samples, $n = 6$, $p < 0.05$) increase of the activation potential by 10 mV on average, what constituted approximately 15% of the initial value of the activation potential. An increase of the Ca^{2+} concentration shifted the activation of the TTX-resistant current by 10 mV towards less negative potential values on average, namely, channel activation required a more depolarised membrane.

Several attempts at recording sodium currents in calcium-free solution (0 mM Ca^{2+}, 0.1 mM EGTA) were undertaken. Recording in calcium-free solution failed because of the quick loss of tightness of the pipette-cell seal at negative pipette potentials.

Discussion

The presented results show that in pyramidal neurons of the prefrontal cortex of rats are present currents which are: TTX-resistant, dependent on sodium ion concentrations, slowly activated and inactivated, with low activation potential. These features are characteristic for sodium current transmitted by the $Na_v1.9$ channels [6]. Expression of TTX-resistant channels ($Na_v1.9$ and $Na_v1.8$) and their activity (presence of TTX-resistant currents with different activation potentials) was already shown before in pyramidal neurons of the prefrontal cortex of rats [8–10]. Thus, our electrophysiological studies provide additional confirmation of the presence of functional, TTX-resistant channels with low activation potential in pyramidal cortex neurons of rats. So far, no proofs confirming the presence of these channels in the prefrontal human cortex are available.

The influence of extracellular calcium on activation of sodium channels is a phenomenon observed for voltage-gated sodium channels, sensitive to TTX [25]. The occurrence of a similar phenomenon in TTX-resistant channels indicates that the activity of calcium ions is non-specific.

The mechanism of extracellular calcium impact on sodium channels has not been clearly explained before. One of the proposed mechanisms is the mechanism in which the membrane potential is shielded by Ca^{2+} ions attracted to the membrane surface by negative membrane charges (e.g. hydrophilic phospholipids and membrane proteins). This mechanism was proposed by Hill [26, 27]. In the case of the proposed mechanism, calcium ions disrupt the operation of the sodium channel membrane potential sensors through neutralization/shielding of membrane anionic groups. However, this mechanism has not been confirmed in studies on the functioning of potassium delayed rectifier K_{DR} channels [26]. The cited work showed that extracellular calcium does not influence the functioning of the K_{DR} channels. Thus, screening of the potential by calcium ions would not apply to the aforementioned channel and it should be a mechanism equally applicable to Na^+ and K^+ channels. A different action mechanism was proposed for Ca^{2+} ions by Armstrong and Cot [28]. They suggested that calcium binds to the channel protein with affinity different in the case of open and closed channel. If the closed channel shows higher affinity to Ca^{2+} than the open channel, a transition between the closed and the open state would be more difficult if calcium binds to the channel. At high calcium concentrations, this would result in a shift of the activation threshold towards depolarization. This model was confirmed for selected channels in studies by Boccaccio et al. [25]. It seems, however, that more studies should be performed to verify the described models.

Despite the fact that we are unable to specify the mechanism of calcium activity influencing TTX-resistant sodium channels, we showed that these are currents susceptible to the concentration of extracellular calcium. A calcium concentration increase of 5 mM results in a shift of the activation potential towards depolarization by 10 mV.

Sodium channels with low activation threshold potentials and slow inactivation do not play a significant role in the conduction of activation potential, however, they are important in the regulation of resting potential and influence cell excitability [6]. The significance of the influence of the observed shift in the activation

threshold of TTX-resistant currents should be explained in studies on the influence of calcium on the resting potential of cells. The resting potential is determined with a significant contribution of other channels, mainly potassium leak channels. It is known that intracellular calcium blocks some of such channels [29]. In addition to the leak potassium channels, sodium leak channels (NALCN) also influence membrane potential. These channels are blocked by extracellular calcium in a G-protein dependent mechanism [30].

Prefrontal cortex plays an important role in planning, initiating and controlling undertaken activities. This area participates on attention focusing and is responsible for working memory [31]. This is confirmed by studies on animals which indicate, that prefrontal cortex neurons control fear-related memory, natural fear and working memory [32–34]. Functional disruptions of the prefrontal cortex are observed in neuropsychiatric conditions, such as depression [35], bipolar disorder [36], anxiety [37], anti-social behaviour disorders [38] and schizophrenia. $Na_v1.9$ channels could influence prefrontal cortex functions through the influence on neural excitability. The presented study contributes to future studies on mechanisms controlling the activity of TTX-resistant channels in the central nervous system. It also indicates that TTX-resistant currents in DRG neurons may also be regulated by the concentration of extracellular calcium.

Conclusions

The experimental study confirmed that TTX-resistant channels are present in cell membranes of pyramidal neurons of the medial prefrontal cortex of rats and may thus play a physiological role in the transmission of sodium currents dependent on the concentration of calcium ions in the extracellular solution.

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ORIGINAL ARTICLE

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Myonectin serum concentration changes after short-term physical activity among young, healthy people

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ABSTRACT

Background: Myonectin is a myokine secreted by skeletal muscles in response to physical activity (PhA) in rodents. It was shown that myonectin may be positively associated with insulin resistance parameters. The aim of the study was to evaluate changes in the concentration of myonectin after short-term PhA.

Methods: A total of 29 young, healthy volunteers, were included in the study. Each participant completed a life-style questionnaire, underwent a physical examination with anthropometric measurement followed by a treadmill test according to theBruce protocol. Blood samples were collected before and after PhA. An ELISA Assay was used to investigate the myonectin serum level.

Results: The myonectin serum level did not change significantly after PhA (0.62[0.14-2.9] vs. 1.08[0.15-2.44] ng/ml; p=0.84). Before PhA the myonectin serum level differed significantly between men and women (respectively: 3.92[2.24-5.30] vs. 0.56[0.15-1.75] ng/ml; p=0.02). Before PhA it had a positive association with weight, BMI, serum creatinine and uremic acid ($p < 0.05$). The change in the level of myonectin serum after PhA had negative associations with weight, BMI, fasting insulin level and HOMA-IR ($p < 0.05$).

Conclusions: Myonectin serum concentration does not change after short-term physical activity among young, healthy people. Changes in the myonectin serum level after short-term physical activity may be associated with fasting insulin resistance.

Keywords: myonectin, physical, activity, insulin, resistance, treadmill, homa-ir

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Introduction

Myonectin (CTRP15/C1QTNF/erythroferrone) is a novel myokine secreted by skeletal muscles in response to physical activity (PhA) and the rise of glucose and free fatty acids (FFA) in rodents [1–2]. Myonectin increases the expression of proteins transporting FFA through hepatocytes and adipocyte cell membranes, causing increasing serum FFA uptake [3]. Toloza et al. reported that myonectin level is associated with insulin resistance (IR) in non-diabetic adults [4]. Lim et al. observed that long-term, regular PhA decreases both the myonectin serum level and IR measured by the homeostasis model assessment [5].

It is known that short-term PhA transiently increases serum levels of glucose, FFA and IR[6]. In this context, it may be suspected that myonectin serum level should increase efter short-term PhA in humans. Currently, no study has yet investigated changes in myonectin serum level after short-term PhA in human adults.

The aim of the study was to evaluate changes in the concentration of myonectin after short-term physical activity.

Materials and Methods

Design

Cross-sectional, single-centre study.

Data Collection

The study group consisted of volunteers, young healthy people. All patients were informed about the study and signed an informed consent form. The study

protocol was approved by the local Ethic Committee (Bioethics Committee of Poznan University of Medical Sciences).

Inclusion and exclusion criteria

All patients who met the following criteria were included: age between 18–26, visitworklab in fasting state, voluntarily signed a consent form. Exclusion criteria were as follows: metabolic syndrome, acute infection, mental disorder, congenital disease, unstable chronic disease, general exclusion criteria for a treadmill test.

Clinical and laboratory data

The worklab visit took place in the morning hours (8:00–10:00 AM). All participants had been informed not to eat, drink or smoke 8 hours prior to attendance. All individuals completed a questionnaire including details of sex, age, smoking status and The International Physical Activity Questionnaire (IPAQ) — a validated questionnaire used to assess weekly PhA expressed as a total weekly metabolic equivalent of the task (MET) [7]. The patients underwent a complete physical examination with anthropometric measurements (weight, height, waist circumference) and blood pressure check. Blood pressure was measured three times by the Korotkoff method in the sitting position, after 10 minutes rest, using a mercury manometer. Blood pressure was additionally measured after 6 minutes of treadmill test. Height and weight were measured using the same medical scales for all patients. Weight was measured to an accuracy of 100 g and height to 0,5 cm. BMI was calculated from the following equations: BMI = weight / squared height [kg/m2].

Blood samples were collected in a fasting state using the S-Monovette blood collection system. Serum glucose, serum insulin, creatinine, uremic acid and lipid profile were assessed according to standard laboratory protocols. After the first blood sample collection and before the treadmill test, participants ate breakfast. 6 minutes after the treadmill test a second blood sample was obtained to assess myonectin serum level after PhA. To estimate myonectin serum concentration Human (CTRP15/Myonectin) ELISA Kit (ElAab, China) was used. All steps of the assay were done according to the manufacturer's instructions. Changes in the myonectin serum level were defined as the difference between myonectin serum level after and before physical activity. HOMA-IR was calculated on the basis of the equation [8]:

HOMA -IR = (Glucose * Insulin)/22.5

Glucose in mmol/l, Insulin in μIU/ml.

Treadmill tests were performed by a physician in accordance with the Bruce protocol on Cardiotest1612 (Aspel, Zabrzów, Poland) [9]. For interpretation of the treadmill test and counting MET the treadmill manufacturer (Aspel, Zabrzów, Poland) software was used.

Statistical analysis

Statistical analysis was performed with STATISTICA 12.0 (StatSoft, USA). The normality of variable distributions was tested using the Shapiro-Wilk test. For descriptive analysis, Wilcoxon test was used to compare the results before and after the treadmill test;the Mann-Whitney was performed to compare men and women. For comparison of binary variables, Fisher's exact test was used. Differences with a p-value < 0.05 were considered statistically significant.

Results

The study group consisted of 29 participants (male = 10; 34.5%), the features of the group are presented in Table 1. All participants had myonectin serum levels within the assay range (0.05–15 ng/ml). To compare myonectin serum levels and other features between men and women the Mann-Whitney U test and Fisher's exact test were used.

Differences between myonectin serum level, heart rate, systolic and diastolic blood pressure before and after physical activity were presented in Table 2. The R Spearman rank correlation test revealed some significant associations between myonectin serum level (Table 3).

Discussion

In this study, no significant change was observed in the myonectin serum level after short-term PhA. Myonectin before PhA was also associated with weight, BMI, serum creatinine and uremic acid. The groups of men and women differed significantly in age, height, weight, BMI, HDL-C, serum creatinine, uremic acid and myonectin before PhA levels. Myonectin is secreted by skeletal muscles. Skeletal muscle mass in young, healthy adults is positively correlated with weight, BMI. Moreover, serum creatinine and uremic acid levels are dependent on muscle mass [10–11]. These associations may provide a rationale for the observed results.

Myonectin in response to PhA increases the expression of CD36, fatty acid transport proteins (FATP), and fatty acid binding proteins (FABP) in hepatocytes and adipocyte, resulting in increasing FFA uptake [2]. It is known that myonectin is associated with IR [4–5] and circulating FFA induces IR [12]. It is possible that myonectin is secondarily upregulated in the IR state in order to diminish circulating FFA levels. In a young,

Table 1. General characteristics of the study group

Feature [units]	median (IQR) / n (%)	Female (n = 19) median (IQR) / n (%)	Male (n = 10) median (IQR) / n (%)	p-value
Smokers	4 (13.8)	2 (10).5	2 (20.0)	0.43
Age [years]	**22 (20–23)**	**21 (20–22)**	**23 (22–23)**	**< 0.01**
Height [m]	**1.72 (16.5–1.76)**	**1.69 (1.64–1.72)**	**1.82 (1.76–1.85)**	**< 0.001**
Weight [kg]	**59 (54–78)**	**54 (53–59)**	**80 (78–86)**	**< 0.001**
BMI [kg/m^2]	**21.3 (19.4–24.0)**	**20.1 (18.5–21.3)**	**24.0 (23.8–25.7)**	**< 0.001**
Fasting Glucose [mmol/]	5.1 (4.8–5.3)	5.1 (4.8–5.3)	5.1 (4.6–5.4)	0.80
Fasting insulin [μIU/ml]	16.1 (12.5–19.1)	16.7 (12.1–18.6)	15.2 (12.8–19.6)	0.79
HOMA-IR	3.7 (2.8–4.3)	3.7 (2.6–4.2)	3.4 (3.0–4.3)	0.98
Total Cholesterol [mmol/l]	4.4 (3.9–5.2)	4.4 (4.2–5.4)	4.0 (3.7–4.6)	0.07
HDL-C [mmol/l]	**1.6 (1.4–1.9)**	**1.7 (1.5–2.1)**	**1.4 (1.2–1.7)**	**0.04**
LDL-C [mmol/l]	2.3 (2.0–3.0)	2.3 (2.0–3.1)	2.2 (1.8–2.5)	0.35
Triglycerides [mmol/]	0.84 (0.61–1.1)	0.85 (0.63–1.1)	0.70 (0.48–0.98)	0.33
Serum Creatinine [μmol/l]	**70.4 (70.3–79.6)**	**70.7 (61.9–70.7)**	**88.4 (79.6–97.3)**	**< 0.001**
Uremic Acid [μmol/l]	**279.6 (243.9–339.0)**	**243.9 (214.1–279.6)**	**358.9 (339.0–395.5)**	**< 0.001**
METmax during Treadmill Test [kcal/kg/h]	11.8 (10.4–14.3)	11.5 (10.4–14.0)	13.9 (11.8–14.8)	0.23
IPAQ Total Weekly MET [kcal/kg/h]	3448 (1794–4730)	3842 (1794–5205)	2669 (1328–3900)	0.11
Myonectin before PhA [ng/ml]	**0.67 (0.14–2.9)**	**0.15 (0.14–1.09)**	**3.92 (2.24–5.30)**	**0.02**
Myonectin after PhA [ng/ml]	1.08 (0.15–2.44)	0.56 (0.15–1.75)	2.43 (0.78–4.00)	0.09
Change of the Myonectin Serum Level	0.01 ([–0.84] – 0.67)	0.01 ([–0.17] – 0.95)	– 0.65 ([–3.38] – 0.67)	0.10

BMI — Body Mass Index, HDL-C — High-Density Lipoprotein Cholesterol, HOMA-IR — Homeostatic Model Assessment Measuring Insulin Resistance, IPAQ — The International Physical Activity Questionnaire, IQR — Interquartile Range, LDL — Low-Density Lipoprotein Cholesterol, MET — Metabolic Equivalent of Task, METmax — Maximal MET

Table 2. Wilcoxon test outcomes

	Before Physical Activity median (IQR)	After Physical Activity median (IQR)	p value
Myonectin [ng/ml]	0.67 (0.14–2.9)	1.08 (0.15–2.44)	0.84
Systolic Blood Pressure [mmHg]	115 (110–124)	120 (112–124)	0.20
Diastolic Blood Pressure [mmHg]	75 (70–80)	72 (70–80)	0.28
Heart Rate [bpm]	82 (78–93)	96 (91–100)	< 0.001

healthy population with no presence of metabolic syndrome, the release of myonectin might be too low and/or slow in comparison with patients with IR to observe any significant change in myonectin serum level in 6 minutes after short-term PhA.

Interestingly, the change in the myonectin serum level after short-term PhA was negatively associated with both insulin in fasting state and HOMA-IR. Toloza et al. studied 81 non-diabetic adults and assessed the association between insulin resistance direct and indirect markers and myokines [4]. Fasting myonectin adjusted to BMI, age and sex were found to be negativelyassociated with the Insulin Sensitivity Index (standardized beta = -0.235, p = 0.023). The authors did not report any significant association with HOMA-IR. Lim et al. investigated the effects of 1-h per week of aerobic physical activity lasting 10-weeks on myonectin and insulin resistance in 14 young (22.5 ± 2.7years) and 14 older (60.3 ± 5.2 years) women [5]. Before the training apositive correlation between HOMA-IR and myonectin serum level was found only in older women in fasting state at rest ($r = 0.35$; $p < 0.05$). After 10-weeks of training, changes in HOMA-IR and changes in myonectin were positively correlated ($r = 0.462$; $p < 0.01$) in both groups. Both the aforementioned studies demonstrated a positive dependence between insulin resistance and

Table 3. Associations between study features and myonectin serum level

Feature [units]	Myonectin Before Physical Activity [ng/ml]		Myonectin After Physical Activity [ng/ml]		Change of the Myonectin Serum Level [ng/ml]	
	Rs	p-value	Rs	p-value	Rs	p-value
Age [years]	0.33	0.08	0.24	0.22	–0.14	0.47
Height [m]	0.30	0.11	0.35	0.07	–0.08	0.68
Weight [kg]	**0.38**	**0.04**	0.21	0.29	**–0.41**	**0.03**
BMI [kg/m^2]	**0.40**	**0.03**	0.19	0.31	**–0.47**	**0.01**
Fasting Glucose [mmol/]	0.07	0.71	0.13	0.50	–0.03	0.88
Fasting insulin [μIU/ml]	0.09	0.64	–0.25	0.19	**–0.38**	**0.04**
HOMA-IR	0.15	0.45	–0.21	0.29	**–0.43**	**0.02**
Total Cholesterol [mmol/l]	–0.01	0.95	0.19	0.32	0.20	0.30
HDL-C [mmol/l]	–0.21	0.28	0.04	0.84	0.21	0.28
LDL-C [mmol/l]	0.13	0.49	0.30	0.11	0.15	0.44
Triglycerides [mmol/]	–0.12	0.55	–0.35	0.06	–0.23	0.22
Serum Creatinine [μmol/l]	**0.40**	**0.03**	0.32	0.09	–0.32	0.09
Uremic Acid [μmol/l]	**0.41**	**0.03**	**0.43**	**0.02**	–0.17	0.38
METmax during Treadmill Test [kcal/kg/h]	0.16	0.41	0.04	0.84	–0.20	0.30
IPAQ Total Weekly MET [kcal/kg/h]	–0.06	0.78	–0.07	0.71	0.01	0.10

BMI — Body Mass Index, HDL-C — High-Density Lipoprotein Cholesterol, HOMA-IR — Homeostatic Model Assessment Measuring Insulin Resistance, IPAQ — The International Physical Activity Questionnaire, IQR — Interquartile Range, LDL — Low-Density Lipoprotein Cholesterol, MET — Metabolic Equivalent of Task, METmax — Maximal MET

myonectin. In our study, no such association was found. However, the negative association between changes in the myonectin serum level after PhA and simultaneously no significant change in myonectin serum level after PhA is intriguing. The change in myonectin serum level is highest among young volunteers with the lowest HOMA-IR value in the fasting state. It is possible that in those groups an increase of myonectin is more rapid than in the group characterized by higher HOMA-IR values. However, this possible phenomenon requires more detailed studies.

This study has several limitations. Firstly, we included only 29 volunteers in the study. Secondly, no additional data such as body composition or FFA were collected. Body composition analysis may reveal a potential association between myonectin serum level and muscle mass. Finally, we have not collected blood samples after a longer period of time than 6 minutes.

In conclusion, myonectin serum concentration does not change after short-term physical activity among young, healthy people. Myonectin serum level may be associated with muscle mass in a young, healthy population. Changes in myonectin serum level after short-term physical activity may be associated with fasting insulin resistance.

Disclosure

None of the authors declared any potential conflict of interests.

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Contribution:

Concept — CM, KarM, Data collection — KamM, JK, GA, SJ, KarM, CM, Laboratory analysis — LB, WA, Statistical analysis — KamM, Draft preparation — KamM, Final approval —KamM, JK, GA, SJ, KarM, LB, WA, CM

List of abbreviations:

FFA — Free Fatty Acids
IPAQ — The International Physical Activity Questionnaire
IR — Insulin Resistance
MET — Metabolic Equivalent of the Task
PhA — Physical Activity

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ORIGINAL ARTICLE

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Effectiveness of Krishna Laddoo in treating malnutrition in anganwadi children

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ABSTRACT

Background: This was a prospective pilot study to estimate the burden of malnutrition in the age group of 2.5–5 years and to evaluate the usefulness of dietary changes in them. This research was conducted by the Department of Paediatrics Krishna Institute of Medical Sciences, Karad in selected Anganwadi at Rethare Bk., This study was undertaken to combat the issue of malnutrition of children's in the age group of 2.5 to 5 years. According to WHO-Moderate Acute Malnutrition is defined by a weight-for-height indicator between -3 and -2 z-scores (standard deviations) of the international standard or by a mid-upper arm circumference (MUAC) between 11 cm and 12.5 cm. Severe Acute Malnutrition is defined by very low weight for height below -3z scores of the median WHO growth standards by visible severe wasting. The children identified with SAM & MAM were started on a high nutrition laddu called the Krishna laddu which was specially designed by the Krishna Institute of Medical Sciences Deemed To be University to cater to the deficiencies in malnourished children. The aim of the study was to determine the effectiveness of Krishna laddu in treating malnutrition in children aged between 2.5–5 years.

Methods: This was a prospective study. Total of 43 children attending the 2 selected anganwadi adopted by the Krishna Institute of Medical Sciences, Karad (aged between 2.5–5 years) were screened for malnutrition and graded according to Severe Acute Malnutrition and Moderate Acute Malnutrition and were given Krishna Laddu on a daily basis. Their anthropometry was serially recorded on a weekly basis over a period of 1.5 years to determine the effectiveness of the Krishna Laddu in improving the nutritional status in the malnourished children.

Results: The prevalence rate of malnutrition was 23% (21% MAM and 2% SAM) in the children aged between 2.5–5 years the given population which was reduced to 8% MAM after their diet was added with Krishna Laddu.

Conclusions: Krishna Laddu proved to be beneficial in the nutritional rehabilitation of malnourished children. The highlights of this research were: 1) early identification of malnutrition 2) prompt initiation of a nutritious diet to improve outcome 3) serial monitoring of these children to plot the outcome. 4) All the ingredients used for making the laddu are easily available in the kitchen. 5) The preparation is low cost, making it feasible for use in any setup.

Keywords: Krishna Laddu, SAM, MAM, MUAC.

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Introduction

Malnutrition kills 5 million children every year. Adequate nutrition is critical to child development, especially in the formative years, from 2.5 to 5 years of as it can shape the child's growth, health and development. At this age, children are particularly vulnerable to growth retardation, micronutrient deficiencies and common childhood illnesses such as malnutrition, diarrhoea, pneumonia and acute respiratory infections. In developing countries, children are vulnerable to malnutrition because of low dietary intakes, high incidence of infectious diseases, lack of appropriate care and inequitable distribution of food within the household. Hence this study is very important.

According to WHO, India contributes to about 21% of the global burden of child deaths. The under-five mortality rate (U5MR) for India in 2011 has been es-

timated as 55 per 1000 live births [1]. Malnutrition is the direct or indirect cause in up to 50% under-five deaths. About 25% of children under-five in the world are underweight, and in India, the underweight prevalence rate is at 43%. Also, the prevalence of wasting in India is about 20%, requiring an urgent response [2–3]. According to the NFHS-3 in the year 2015–2016, the trends in children's nutritional status in India was 38% were stunted (too short for age), 19% were wasted (too thin for height), 46% were underweight (too thin for age) [4]. These figures are certainly alarming and warrant intervention.

The Krishna Laddu Project is a well-intentioned attempt at improving the health of children living in a rural background and also for peoples living in poor condition. The approach is innovative, though there have been similar projects and locally focused. Additionally, the project design is largely solid and well thought out. The laddus are a well-chosen and thought-out supplement, they reduce the deficit in the child's nutrition by giving a significant supplement to both the calorie and protein intake. Their simple recipe (requiring minimal cooking time) and long-lasting nature make them very well suited to storage. Additionally, the use of readily available, cheap ingredients is excellent. Also, the dietary counselling contributed to the improvement in the nutritional status of the children.

Materials and methods

The project was carried out over a period of 18 months with their weekly follow-up. A team consisting of the paediatric resident, houseman and a nurse visited 2 Anganwadi's at Rethare Bk. weekly to serially record the weight, height and MUAC of the children between 2.5–5 years who were enrolled at their Anganwadi.

Anganwadi is a novel concept by the Government of India which serves as the first informal education medium and a day care centre for children in this age group of 2.5–5 years. The children were then categorised into SAM/MAM according to WHO criteria.

Krishna Laddu, an innovation of the Krishna Institute of Medical Sciences, Karad was given to each of the SAM/MAM children daily. Friday of each week was chosen by for the anthropometry measurement (weight recorded weekly and height and mid-upper arm circumference recorded every month) and for handing over the laddus for coming week to the health workers at the Anganwadi. The progress of the children was plotted on the WHO growth charts to note the changes in the anthropometry.

The laddoo was a supplement that the children received in addition to the meal consumed at the anganwadi. Each day of the week had different meals like dal khichadi, upma, kheer etc. prepared by the anganwadi workers so as to give about 300 calories and 8-gram proteins to the child.

The laddoo scored better than these meals as the children equated the idea of getting a laddoo to a treat and also because the laddoo, if not finished by the child in one go, could be stored and offered again after a while. This could not be done with the meals as they would turn cold and would not be appetizing to the child anymore.

The ingredients of the laddu were dry roasted before using them. This increased the shelf life of the laddu. Also, at the anganwadis , the laddus were stored in air tight containers to prevent the moisture from making them less palatable to the children. This contributed to keeping the taste and texture of the laddus unaltered for about a week.

The project also served the purpose of a health check-up by the pediatric resident on weekly basis. Issues like de-worming, need for sanitation and hygienic situation, faulty cooking practices, advice with regard to child rearing and nutrition were addressed on the 4th Friday of each month by holding a meeting with the parents under the project. The most important part of these sessions was the dietary counselling regarding a balanced diet and educating the mothers about what diet could help in proper nourishment of their children.

It is worth mentioning here that during the course of the study, the anganwadi workers were interviewed

Contents of the Krishna Laddu

Ingredient	Weight (gms)	Calories (gms)	Proteins (gms)	Carbs (gms)	Fat (gms)	Calcium (gms)	Iron (gms)
Soyabean powder	15	64.8	4.85	2.85	2.95	36	1.56
Ragi powder	05	16.4	0.36	3.6	0.06	17.2	0.915
Chana dal powder	05	18.0	0.85	0.28	0.26	10.1	0.23
Groundnut	05	28.2	2.0	0.27	3.71	4.42	0.31
Jaggery	20	76.6	0.08	19	0.02	16	2.28
Groundnut oil	10	90	–	–	10	–	–
Total	60	294	10	26	17	84	4.575

in depth about the food habits of these children. They said that many parents took the meals provided at the anganwadis and the additional laddoo provided by us as a substitute for the home meals. This was a major fallacy as the child was deprived of their meals at home. The mothers were explained again that this was only a supplement and not a substitute for the food that the child received at home.

Ethical Considerations

As an intervention study involving children, the Laddu Project was presented before the Ethical Committee and permission was obtained from the Ethical Committee for the same.

Statistical Analysis:

Descriptive statistics were used to describe the age and sex distribution of the children. We created binary outcome variables for the diagnosis of severe wasting using MUAC and Weight for height Z scores as described in the methods section. Proportions and 95% confidence intervals were computed to allow comparisons between indicators. The ages of the children were dichotomized to correspond to categories usually used in field nutrition programmes: young (age < 24 months), and older children (age ≥ 24 months). The prevalence percentage was calculated as per cut-off points of WHO & NFHS. The association was calculated using ANOVA & Student t-test. Data were analysed using SPSS 20.0 for Windows statistical package for social science (International Business Machines Corporation, INDIA).

Results

43 children enrolled at the 2 anganwadis , adopted by KIMSDU , were included in the study, age group was of 2.5–5 years i.e. 30 to 60 months, maximum 21 (49%) children's were in the age group of 51–60 months, 13 (30%) children's were in the age group of 41–50 months and 9 (21%) children's were in the age group of 31–40 months. According to sex out of 43 (100%) children's max. 24 (56%) were girls and 19 (44%) were boys which is shown in Table.1 and Figure 1. and 2.

Table 1. Age & sex wise distribution of demographic variables

Demographic Variables	No. of children's	%
Age in Months		
31–40	9	21
41–50	13	30
51–60	21	49
Sex		
Boys	19	44
Girls	24	56

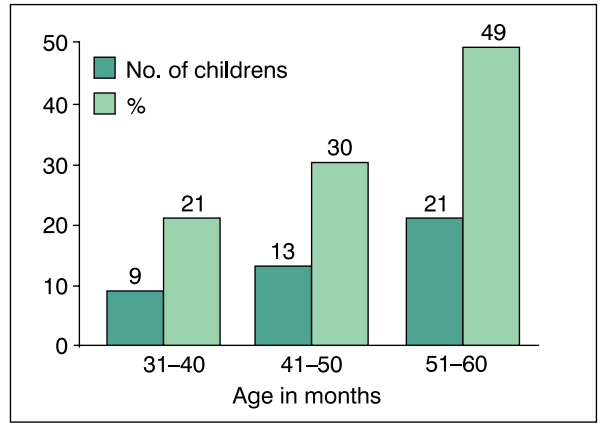

Figure 1. Graphical distribution of age wise distribution

Here, the percent prevalence in overall 43 (100%) children's was calculated as per age group of 31–40 months, 41–50 months & 51–60 months also as per stunting < -2SD (Moderate Class) 52.2%, 53.5%, 52.7% respectively, as per stunting < -3SD (Severe Class) 21%, 22.2%, 21.5% respectively, as per wasting < -2SD (Moderate Class) 10.1%, 10.4%, 8.4% respectively, as per wasting<-3SD (Severe Class) 0.7%, 0.5%, 0%, as

Table 2. Prevalence of Undernutrition among Rural Pre-school Children's

Age Group (in months)	No.	Per cent Prevalence of Undernutrition					
		Stunting (Low height for age)		Wasting (Low weight for-Height)		Underweight (Low weight for age)	
		<-2SD	<-3SD	<-2SD	<-3SD	<-2SD	<-3SD
31–40	9	52.2	21	10.1	0.7	4.7	2.3
41–50	13	53.5	22.2	10.4	0.5	7	0
51–60	21	52.7	21.5	8.4	0	9.3	0

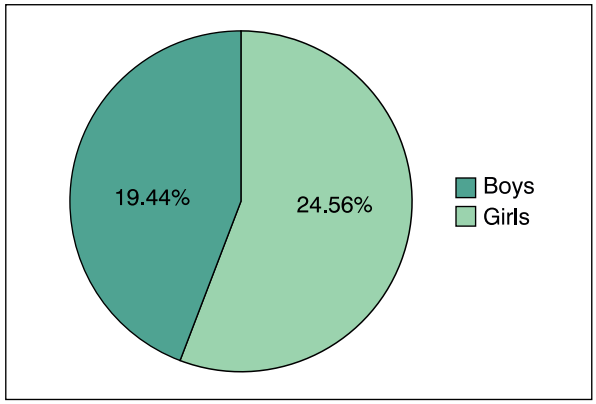

Figure 2. Graphical distribution of Sex wise distribution

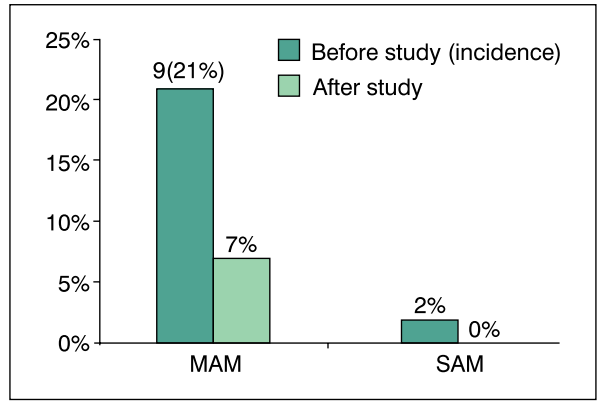

Figure 3. Graphical representation of study prevalence

per underweight < -2SD (Moderate Class) 4.7%, 7%, 9.3% respectively, as per underweight < -3SD (Severe Class) 2.3%, 0%, 0% respectively. Shown in Table.2

Among all 43 (100%) children's maximum 4 (9%) children's age group of 51–60 months were found in MAM, 3 (7%) children's with age group of 41–50 months were found in MAM and 2 (5%) children's were found in MAM and only 1 (2%) with age group of 31–40 months were found in SAM. The prevalence of moderate acute malnutrition was 21% and severe acute malnutrition was 2%. Shown in Table 3.

Association between age group vs. different study variables such as HAZ, WAZ and MUAZ was shown in Table 4. Association between sex and different study variables such as HAZ, WAZ and MUAZ was shown in Table 5.

Table 3. Distribution of prevalence of malnutrition

Age-Group (in months)	No.	No Malnutrition (%)	Moderate Acute Malnutrition (%)	Severe Acute Malnutrition (%)
31–40	9	6(14)	3(7)	1(2)
41–50	13	10(23)	2(5)	0(0)
51–60	21	17(40)	4(9)	0(0)
Total	43	33(77)	9(21)	1(2)

Table 4. Association between different age groups

Study Variable	Mean Z-Score			Standard Deviation			F-value	P-value	Inference
Age Group (in months)	31–40	41–50	51–60	31–40	41–50	51–60			
HAZ	–1.71	–2.4	–2.1	1.28	1.42	1.5	0.6161	0.5451	NS
WAZ	–1.41	–1.79	1.1	1.2	1	0.88	41.586	<0.0001	S
MUAZ	–1.63	–1.57	0.87	0.9	0.87	0.67	0.01454	0.9856	NS

Table 5. Association between different sex groups

Study variable	Mean Z-Score		Standard deviation		t-value	P-value	Inference
Sex	Boys	Girls	Boys	Girls			
HAZ	–2.08	–1.78	1.23	1.5	4.403	0.0001	S
WAZ	–1.49	–1.53	0.97	0.49	-2.928	0.0055	S
MUAZ	–1.59	–1.52	0.81	0.65	0.7176	0.47	NS

Table 6. Prevalence of malnutrition among study population

Prevalence	MAM	SAM
Before Study	21%	2%
After Study	8%	0%

Discussion

The results of this study are deduced and they are discussed below. They are divided into criteria of relevance, effectiveness, efficiency, impact and sustainability. 'Relevance' considers how well the Laddu Project is suited to tackling malnutrition in the Rethare Bk. context. 'Effectiveness' focuses on the goals achieved by the Laddu Project. 'Efficiency' considers whether the project's method is efficient in its processes. 'Impact' looks at the wider effect that the project can have on the community. Finally, 'Sustainability' covers the project's longevity and potential expansion.

Likewise, similar studies have been done in India and outside India which is very important for the assessment of malnutrition**.** In a cross-sectional study, of 309 Kamar Tribal Children aged 4–12 years to assess the nutritional status [6]. The result was more than 90% of children in the age group 4–12 years suffered from underweight which was comparatively lower in 7–9 and 10–12 years age group children. 84.51% percent of boys suffered from stunting which was much higher than girls (47.54%) [6]. The study concluded there was a widespread prevalence of malnutrition among tribal children and an urgent dietary intervention programme was necessary [7].

Another study was community-based and a cross-sectional survey carried out in preschool children of the Gond community in Madhya Pradesh. The result of the study showed more than 60% of children were underweight. Unhygienic personal habits and adverse cultural practices relating to child rearing, breastfeeding and weaning were also prevalent among them [8]. These factors were thought about in the initiation of our study and counselling was effectively carried out to make the mothers aware of the right child-rearing practices.

A total of 6531 Punjabi children in the age group 1–5 years were measured in a cross-sectional study for assessing malnutrition. The percentage of children who were underweight, stunted, wasted and having low MUAC for age was 15.04%, 11.42%, 10.76% and 38.52% respectively [9].

Of late, there has been intense debate and discussion on how to best intervene to make a change that is both substantial and rapid and various groups of experts have presented strategies to policy makers as to the steps that need to be taken for both preventing malnutrition and treating its most severe forms [10]. Many locally produced/producible foods that are culturally acceptable and relatively low cost have been used for severe malnutrition cases in India for many decades by reliable academic and medical institutions as well as by non-governmental groups. One of the effective measures was the use of Leaf Protein Mix to improve the protein intake of children affected with Malnutrition [11].

A study on the effects of curd (dahi) and leaf protein concentrate in children with protein-energy malnutrition. The study was conducted in a tertiary hospital at New Delhi, in which 80 moderately and severely malnourished children of age 1–5 years were given either the curd or LPC in addition to the WHO recommended two-step diet for 15 days. The results revealed a change in weight, haemoglobin level and CD4: CD8 and T-cell subpopulation was significant in both the groups after supplementation. The study concluded that curd and LPC, when added to the diet of malnourished children, would accelerate the immune recovery and improve the nutritional status of the affected children [12]. Our study was also based on maximum utilisation of locally available products making it extremely cost effective.

A study done on the health and nutritional status of preschool children of Amritsar district of Punjab were studied. A total of 1,000 children formed the group. Out of the 1000 children examined, 255 (25.5%) children were found to be suffering from protein-energy malnutrition. 25.18% of boys and 25.85% of girls had protein-energy malnutrition [13].

Studies have also shown that the formula advised by the National Institute of Nutrition, Hyderabad specially prepared protein mixtures provided an increase of weight after 22 days to 3 weeks or little later [14].

How does one achieve a balanced diet with plenty of protein where animal protein sources or even legume seeds are too expensive? The Krishna Laddu mix which can be easily made from the local produce is an outstanding source of quality protein and vitamins. It can be made with very little cost, even from ingredients available in the kitchen.

In view of the above facts, we observed that protein-energy malnutrition is one of the 'silent emergencies' seen in children of age 0–5 yrs of age. The treatment lies in providing malnourished children with energy and protein-rich foods so that there is adequate weight gain. These foods can be prepared from the usual home diets. The home diets may provide insufficient energy and protein to the child leading to further deterioration in the child's nutritional status.

A study was conducted at Delhi regarding malnourishment among the children aged 6 months to 2 years. A total of 1661 children who attended the well baby clinic of UHC, Gokulpur, Delhi during the year

2000 were studied. The results of the studied showed a total of 1009 (60.7%) children were found malnourished [15].

A cross-sectional study was done to assess the health and nutritional status of children of 0–5 years age group and to study the influence of various epidemiological factors on health and nutritional status. The study was carried out in randomly selected three wards of Petlad town, district Anand in Gujarat state. The prevalence of underweight was 43.6%. 50.3% of children were found stunted. Prevalence of wasting was comparatively low of 23.2% [16].

A study carried out in three backward states, two blocks were selected in each state on the basis of the maximum concentration of the backward community. a total of 80 samples were selected from the 18 blocks making a sum of 1440 samples. 66.2% of all children of 0–5 years of age were underweight of which 23.1% were under grade III and IV, showing the severe extent of malnutrition. The highest number of children in Grades III and IV were from Uttar Pradesh 27.1% followed by Orissa 22% and Rajasthan 21.1%. The study also revealed the children were short for age or stunted 32.9% in the age group below three years [17].

A comparative study to determine the biochemical nutritional indicators among children suffering from protein-energy malnutrition and among the well-nourished children of age group 0-5 yrs at outpatient department and wards of Kanti Children's Hospital, Maharganj Kathmandu, Nepal, and the sample was of 120 children. The results revealed that there were altered biochemical parameters which were related to food intake and biochemical metabolism mandatory during growth and development of children less than five years of age [19].

All of the above studies only substantiate the result of our study and add to the importance of home-based supplements.

Conclusion

The anthropometry showed a significant improvement in the health status of the children who received the Krishna Laddu as a supplement. The Krishna Laddu reduced the calorie and protein deficit in the malnourished children. Krishna Laddu proved to be beneficial in the highlights of this research were: 1) early identification of malnutrition 2) prompt initiation of a nutritious diet to improve outcome 3) serial monitoring of these children to plot the outcome. 4) All the ingredients used for making the laddu are easily available in the kitchen. 5) The preparation is low cost, making it feasible for use in any setup.

Additionally, the counselling of the mothers helped in better understanding of hygiene and nutrition.

It would be very interesting to investigate how exactly the Laddu Project could aid the supplementary feeding programme of the ICDS. The laddu could be used as an intervention for nutritional rehabilitation of the malnourished children in a large community too.

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REVIEW ARTICLE

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Plant stem cells culture — a new tool for skin protection and regeneration

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ABSTRACT

Development of biotechnology, esthetic medicine and cosmetology can enable us to slow down or delay the skin aging process. Currently, much attention is aimed at treatments using substances of plant origin. They have been proven to exhibit antioxidant, antibacterial and antifungal properties, accelerate wound healing, moisturize the skin, enhance skin renewal processes and protect skin against UV radiation. Biologically active plant-derived compounds, however, are often produced by plants in very small amounts. A solution to this problem is an *in vitro* culture of callus tissue, representing plant stem cells. Both, *in vitro* and *in vivo* studies demonstrated beneficial effects of plants stem cell extracts on human skin in the battle against ageing. The aim of this paper was to provide a review of studies based on the use of plant stem cells in limiting skin ageing.

Key words: phytotherapy; plant-derived compounds; plant stem cells; skin ageing

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Introduction

The skin is the largest organ of the human body and performs many physiological functions, including protection against the adverse effects of external factors, body temperature regulation, secretion and absorption of various agents [1–3]. The skin consists of a number of specialized cells forming three layers: epidermis, dermis and subcutaneous tissue. Each layer plays a different, but very important role in maintaining the proper functions of the entire body [1]. Homeostasis of the skin (all of its layers) is possible by the presence of various stem cell populations located mainly in the epidermis (interfollicular compartments, the bulge region of the follicle, sebaceous glands). Their presence and condition determine regeneration and skin cell renewal while maintaining a balance between all layers [4]. It also allows for maintaining a healthy-looking skin, which is an attribute of each person. Unfortunately, over time skin undergoes an inevitable ageing process which is irreversible and affects every human being. Skin ageing is a result of overlapping multiple mechanical, biological, biochemical and molecular processes, which begins around age 25–30. Depending on the causes, ageing can be both intrinsic and/or extrinsic [5–7]. The first one is determined genetically and hormonally and is highly time-dependent. The second one, called also photoaging, is caused by various external factors affecting the skin (eg. UV radiation, cigarette usage, pollution and stress [5–8].

However, thanks to advances in many areas of biotechnology and medicine, it is possible to slow down or significantly delay this complex process. Properly selected nutrients as well as cosmetic care are crucial in the renewal and regeneration of skin and increasing its resistance to external factors such as sudden changes in temperature, strong wind, pollution and UV radiation. Currently, a great amount of attention is paid towards treatments that may delay skin ageing and/or eliminate visible symptoms of this process. Among cosmetics based on natural raw materials (such as creams and lotions), cosmetological treatments (including facials and infusions), as well as various skin injections, surgery and laser treatments, are used. Treatments with various kinds of cosmetics (creams, facials) that contain a number of biologically active substances having anti-ageing, anti-wrinkle, bleaching properties, etc., are commonly applied due to their ease of use and low risk of complications or adverse side effects. For years, those types of cosmetics, great importance was assigned to regenerative properties of plants. They contain ingredients that are necessary to carry out all metabolic processes responsible for the renewal of the epidermis, increasing the skin elasticity, scavenging free radicals and maintaining an adequate level of hydration.

The aim of this review was to provide an overview of the studies concerning the use of plant stem cells in the case of limiting the skin ageing.

Plant compounds in cosmetics

Plants are a natural resource, used for centuries for cosmetic and therapeutic purposes. There are many cosmetics, of which the active compounds are the main base. These compounds are obtained by extraction with various kinds of solvents from selected plants. They affect preferably the condition of the skin and prevent the loss of elasticity by stimulating synthesis of collagen and elastin fibres. They also exhibit antioxidant, antibacterial and antifungal properties, accelerate wound healing, moisturize the skin, enhance skin renewal processes and protect skin against the harmful effects of UV radiation [9]. There are a number of substances of plant origin which positive effects on the skin were confirmed. These are vitamins, unsaturated fatty acids, saponins, phytohormones, flavonoids, alkaloids and carotenoids.

Vitamins

Vitamins serve as antioxidants, support the renewal of damaged collagen and elastin fibres. Vitamin C serves one of the most important roles, it is a potent antioxidant that protects skin from free radicals created inter alia by UV radiation — photoprotection [10, 11]. Vit. C plays also a key role in the synthesis of collagen [11, 12] since its presence is necessary for the proper functioning of proline hydroxylase and lysine hydroxylase. These enzymes are responsible for the formation of the correct collagen molecule, the main protein responsible for maintaining the skin mechanical properties [12–14]. There have also been reports demonstrating the specific role of tocopherol (vitamin E) in scarring and wound healing processes [15]. This compound is also involved in the stabilization of biological membranes and, is also a strong antioxidant. Its deficiency, in turn, can severely accelerate the aging processes [14]. Fruits and vegetables are a rich source of vitamins [10], ones with a high overall vitamin content are primarily: rosehips [16, 17], blackcurrant [18, 19], chokeberry [19], tomatoes, kiwi [20], avocado [21], strawberries, papaya and broccoli [11].

Unsaturated fatty acids

Unsaturated fatty acids are one of the components of the most external layer of the epidermis (*Stratum corneum*), and it's built of corneocytes [22, 23]. They increase the flexibility of cell membranes, help to maintain an adequate level of hydration and skin tightening, enhance the epidermal barrier function and support defensive processes of the skin. A deficiency of these components in the skin, may cause severe skin diseases such as eczema [24]. It also leads to the reduction in hydration, which in turn results in reduced flexibility and accelerated the formation of wrinkles [21, 25]. Due to the presence of multiple bonds unsaturated fatty acids exhibit antioxidant activity, reducing the risk of formation of free radicals that accelerate the ageing process and can lead to skin cancers. Unsaturated fatty acids are achieved mainly from vegetable oils contained, inter alia, in black cumin seed [26], avocado [27], primrose [28] or hemp oil [25].

Saponins

Saponins are a group of compounds belonging to the glycoside. They exhibit properties similar to soap since they have the ability to decrease the surface tension and cause the foaming process [29]. These make them useful in cosmetic products designed for skin cleaning and toning such as tonics, make-up removers or cleaning gels. Another, very important, the feature of saponins is the ability to penetrating through the lipid layer of the skin due to the presence of a lipophilic aglycone in their structure. This property facilitates the absorption of other active substances contained in cosmetics by increasing membrane permeability [30]. Scientific studies have shown that saponins applied to the skin exposure to UV radiation, inhibit the appearance of wrinkles and increasing the thickness of the skin and loss of their elasticity. This made them highly effective photoprotective compounds [30]. Saponins are mainly found in the leaves of plants such as ginseng [30], acacia [31], soybeans and spinach [29].

Phytohormones

Phytohormones serve an excellent alternative to the human hormones (such as estrogen) which are used in many types of beauty products. Scientific studies have shown the positive effects of estrogen skin on regeneration. It also decreases the aging process [32, 33]. However, the use of such compounds has been banned because they caused too many side effects, unlike their plant counterparts [34]. Plant hormones prevent cell membranes damage and its oxidation. Phytohormones exhibit inhibitory activity of collagenase, elastase and hyaluronidase — enzymes that cause water loss and reduce the skin elasticity, making it more susceptible to the aging processes [35]. In *in vivo* studies, a cream containing phytohormones caused a reduction in wrinkle depth after a few weeks of use [36]. In addition, it has been shown that phytohormones can reduce the deposition of fat so can be successfully used in cosmetics that modelling the face oval. Common sources of these compounds are grapefruit, ginseng, dates, soybeans or wheat [35].

Flavonoids

Flavonoids are applied in skin care products mostly due to its powerful antioxidant properties, which can be up to 100 and 25-times stronger than vitamin C and vitamin E, respectively [35]. Furthermore, they accelerate wound healing, inflammatory and antibacterial processes. Provide also an excellent barrier to UV rays. That is why they can be used to reduce the natural skin ageing process and protect it from the damaging effects of free radicals [37]. In an indirect way, flavonoids affect the condition of the blood vessels in the skin by stabilizing the unoxidized form of vitamin C, which is responsible for maintaining their cohesiveness. It was also shown that flavonoids inhibit elastase and hyaluronidase activity [35]. Flavonoids derived mainly from citrus fruits, tomatoes, vegetables, legumes, broccoli, blueberries, bilberries and grapes.

Alkaloids

Alkaloids which are metabolites of many plants are now also used in cosmetic preparations. The best-known and most frequently used are caffeine, quindoline, allocryptopine. Caffeine extracted from seeds of *Coffea arabica* is the most common, due to the ability to penetrating the skin very effectively [38]. It is also reported that caffeine has the ability to reduce the risk of skin cancer, after exposure to UV radiation [39]. Furthermore, caffeine is also often used in firming, anti-cellulite and slimming cosmetics due to its lipolytic properties [40].

Carotenoids

The use of carotenoids in cosmetics is less popular than the above-mentioned compounds, but their protective properties for the skin are also very important. The greatest source of lycopene and β-carotene — the main representatives of carotenoids — are commonly available in red vegetables, such as tomatoes, peppers and carrots. Carotenoids have an antioxidant property and are therefore widely used in cosmetics which are applied against the UV radiation. It also has shown a high antitumor property for various types of cancers [21].

Obtaining plant derived-compounds

Plant-derived biologically active compounds used in cosmetology and aesthetic dermatology are mainly secondary metabolites. Their receiving from natural plant sources is often a complex, time-consuming and usually inefficient process. This is mainly due to the fact that secondary metabolites are produced in plants in an amount less than 1% of dry weight [41]. Therefore, obtaining of appropriate quantities of desirable compounds requires their isolation from a large amount of plant material. This factor is extremely limited due to the presence of geographical barriers; variability of the seasons, which is bound with the growth of the majority of plant species, as well as various culture conditions that may hinder the obtaining plant material. The problem is also protected species of plants which use, even for scientific studies, require the appropriate permits [9, 41]. On the other hand, obtaining of plant compounds by chemical synthesis is often impossible, because they have usually very complex structure (polycyclic, containing a plurality of chiral centers) [41].

A solution of the above problems came with the intensive development of biotechnology and *in vitro* culture of plants [9, 42]. They allow a culture of selected organs or plant cells in solid, controlled conditions, reducing the time necessary to obtain a large amount of material, in relation to the whole plant breeding. In recent years, particular interest is focused on *in vitro* cultures of callus tissue, as a plant stem cells. Callus is a plant tissue resulting from the undifferentiation of adult plant cells at the site of mechanical injury. To initiate this type of the culture even a small explant of the plant can be used. This allows obtaining a large amount of material (biologically active substances) in the relatively short time, without breeding of the whole plant. Therefore, the stem cells of plants are an alternative to obtaining the biologically active compounds from selected plants.

Plant stem cells

Plant stem cells (PSCs) are the source of a number of factors supporting the skin protection and regeneration processes and thereby inhibit skin ageing. The first reports about the possibility of using stem cells derived from plants in cosmetic products have appeared in 2008 [43]. Extracts from stem cells such plants as apple (*Malus domestica*) varieties Uttwiler Spätlaubersą, argan (*Argania spinosa*), alpenrose (*Rhododendron ferrugineum*), grape vine (*Vitis vinifera*), samphire (*Crithmum maritimum*) and tomato (*Lycopersicon esculentum*), have been analyzed so far. Both, i*n vitro* and *in vivo* studies demonstrated beneficial effects of stem cell extracts of above standard plant extracts on the skin. However, PSCs derived from different plants have varying biological properties.

Stem cells derived from apple fruit and argan stimulate the proliferation and survival of human stem cells [43, 44] and in the case of apple PSCs — protect them from the adverse effects of UV radiation [43]. These results suggest that stem cells derived from plants

Table 1 Clinical trials of plant stem cells extracts on skin regeneration

Stem cells source	Participants	Application	Results
Apple (*Malus domestica*) — clinical trial (Schmid et al. 2008; Schmid 2009)	20 women age: 37–64 years	cream with 2 % cell extract; two times a day for 4 weeks	— significant reduction of wrinkles by 8% and 15 % after 2 and 4 weeks respectively.
Argan (*Argania spinosa*) — clinical trial (Montaño 2012)	21 women age: 39–61 years	emulsion with 0,4 % cell extract; two times a day for 56 days	— reduction of wrinkles by 19 % and 27 % after 28 and 56 days respectively. — increase in skin density of 12.7 % after 8 weeks
Samphire (*Crithmum maritimum*) — comparative intra--individual, controlled, randomized pilot study (Caucanas et al. 2011)	12 volunteers age: older than 50 years	— cream with 0.05 % cell extract, — cosmetic serum with 0,1 % cell extract, — silicone oil with 0,5 % cell extract once a day for 2 weeks, observations for another 2 weeks	— improvement of skin permeability barrier function by: significant decreases in transepidermal water loss (TEWL), between day 16 and 29; smoothing of the skin between day 15 and 29; significant skin moisturization at day 29 compared to day 1 — no adverse events were observed

can contribute to both, protection and regeneration of the skin. Additionally, PSCs extract from apple fruit also showed antioxidant properties by stimulating the expression of genes which play an important role in the regulation, cell growth and proliferation (cyclin B1 and E1, p53, IGF II, heme oxygenase 1) [43]. Samphire stem cells extract have a positive influence on the proliferation and viability of human keratinocytes and fibroblasts [45]. Tomato stem cells, in turn, exhibit a protective effect on murine keratinocytes and fibroblasts cells during oxidative stress induced by certain heavy metals (Ni and Pb). It stimulated the production of collagen I and III by fibroblasts in the presence of heavy metals and decreased the expression of collagenases: MMP 1, 3 and 9, which rises in the above conditions [46]. In *in vivo* studies using human skin model (SE, skin equivalent model) suggest the increase of cell proliferation in the presence of PSCs, samphire extract. This resulted in the growing thickness of the individual skin layers and increased cell density in both the epidermis and dermis [45]. In clinical studies, extract of stem cells from samphire improved epidermal permeability barrier repair and revealed the soothing and moisturizing effect on the skin [47]. The extracts of apple and argan stem cells, in turn, reduced wrinkles appearing around the eyes ("crow's feet") and resulted in an increase of skin density [43, 48, 49]. Detailed information about clinical trials of plant stem cells applications are presented in Table 1.

Conclusion

The continued desire of society to maintain a healthy, youthful appearance forced to search for novel ways to inhibit and even reverse the ageing processes. There are many rejuvenating treatments that restoring the skin's youthful appearance, ranging from simple creams application and plastic surgery in the ending. Although the invasive methods give the most spectacular effects, in a relatively short period of time, they are often painful and carry a risk of side effects (scars or wounds difficult to heal). Therefore, researchers are currently focused on the search for natural methods to fight with the signs of unavoidable skin ageing. One of such methods is the use of plant-derived compounds in cosmetic and dermo-cosmetics.

The plants are a very rich source of many biologically active compounds, which are used in many areas of our lives. Studies confirm the beneficial effects of many substances of plant origin on the functioning and protection of the skin. Their use in cosmetics and personal care products protect the skin against the damaging effects of external factors, promotes skin regeneration processes and inhibit and/or slow down the ageing process. This allows maintaining a healthy, firm skin for many years. The use of plant compounds provides many advantages. As these are natural compounds, they rarely lead to undesirable side effects. Furthermore, they are not complex in use and available to all, because of the form of its administration — mostly creams, masks and lotions.

Currently, in addition to the standard compounds of plant origin, much attention is also paid to the plants stem cells. They appear to be a promising material for use in cosmetology and aesthetic dermatology due to its protective and regenerative properties. Studies, conducted so far, confirmed the potential of these cells in the fight against the signs of skin ageing, emphasizing their beneficial effects on stem cells, which is crucial to the process of skin renewal.

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VIA MEDICA

CASE REPORT

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Biphosphonates-related osteonecrosis of the jaw

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ABSTRACT

The relationship between osteonecrosis of the jaw and bisphosphonate therapy has been described recently. Although bisphosphonates have a long list of benefits in the treatment of patients with bone metastases, an increasing number of reports describe the complication of bisphosphonate-related osteonecrosis of the jaw (BRONJ). Bisphosphonates are synthetic analogues of pyrophosphate that inhibit bone resorption. The aetiology of BRONJ is unclear. It starts as aseptic necrosis, with a surgical procedure involving an interruption in the continuity of the oral mucosa as an obligatory precondition. Subsequently, areas of osteonecrosis occur and detach from the surrounding areas of purulent inflammation or can be removed surgically. Due to the limited treatment options, conservative treatment supported by antibiotic and surgical therapy is used. This article describes a case of BRONJ.

Key words: bisphosphonate, osteonecrosis, jaws, osteoporosis

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Introduction

Bisphosphonates are medicines useful in patients with bone metastases originating in primary breast, prostate and kidney cancers, multiple myeloma and Paget's disease [1]. Bisphosphonates are also used by patients with osteoporosis [2, 3].

The multi-directional activity of these drugs with their inhibitory effect on bone resorption has many benefits but is also burdened by adverse effects [4]. A particular adverse effect affecting the oral cavity is bisphosphonate-related osteonecrosis of the jaw (BRONJ).

It is defined as an area of exposed bone in the maxillofacial region which does not heal within 8 weeks in a patient currently or previously treated with bisphosphonates and not subjected to radiation therapy of the head or neck [5, 6, 7].

The disease is mainly associated with intravenous administration of high doses of bisphosphonates, but cases of BRONJ in patients treated with low oral doses have also been observed [8]. Treatment and prosthetic rehabilitation of the affected patients are difficult and very limited.

Case description

A patient receiving treatment for breast cancer. In 1997, right-side mastectomy was conducted due to cancer, followed by chemotherapy and radiation therapy. In 2002, a local recurrence was detected along with metastases to the spine and the supra- and infraclavicular lymph nodes on the right side. In 2005, metastases to the shoulder tissues were detected. Type 2 diabetes mellitus. The patient was treated with bisphosphonates between July 2002 and May 2017. Treatments used include, inter alia, clodronic acid, 90 mg every 4 weeks, i.v. (Jul 2002–Oct 2005), pamidronic acid, 60 mg every 4 weeks, i.v. (Nov 2005–Feb 2011), zoledronic acid, 4 mg every 4 weeks, i.v. (Mar 2011–May 2017). In 2011, the patient underwent maxillary tooth extractions. The alveoli were not closed with stitches. Not all post-extraction wounds healed, and osteonecrosis occurred in the incisor area. The patient was hospitalized several times due to pain in the area. In January 2018, maxillofacial CT with contrast revealed "loss of the right-side alveolar process of approx. 30×16×17 mm without separation of pathological mass — necrosis?" (Fig. 1).

In April 2018, the patient reported pain in the area and leakage of fluid through the nose during drinking. In physical examination, exposed right-side maxillary alveolar process between the alveoli for teeth 11 to 13, reaching the vestibular fornix (Fig. 2). Increased oral hygiene recommended.

Discussion

In terms of chemical structure, bisphosphonates are synthetic analogues of pyrophosphate (natural

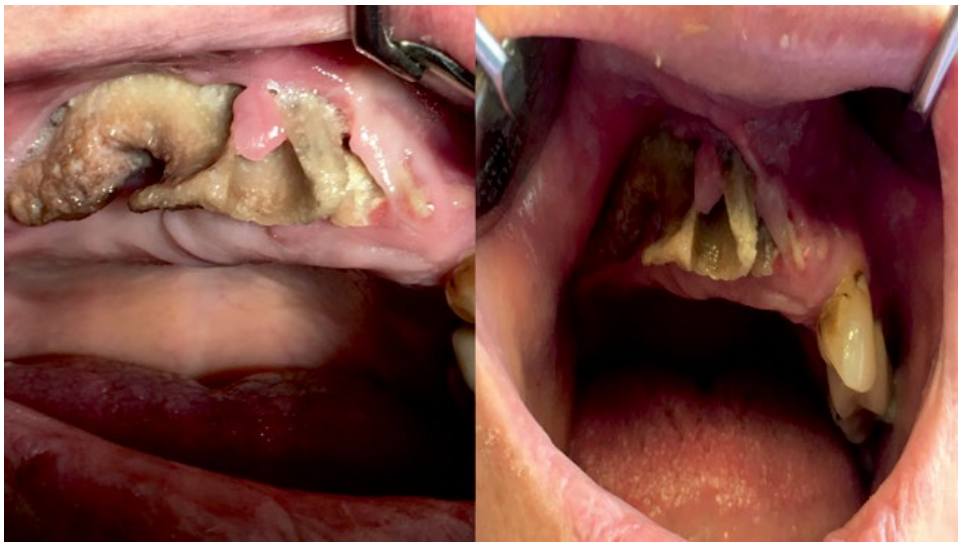

Figure 1. Current CT image — red arrows indicate a bone loss in the maxilla

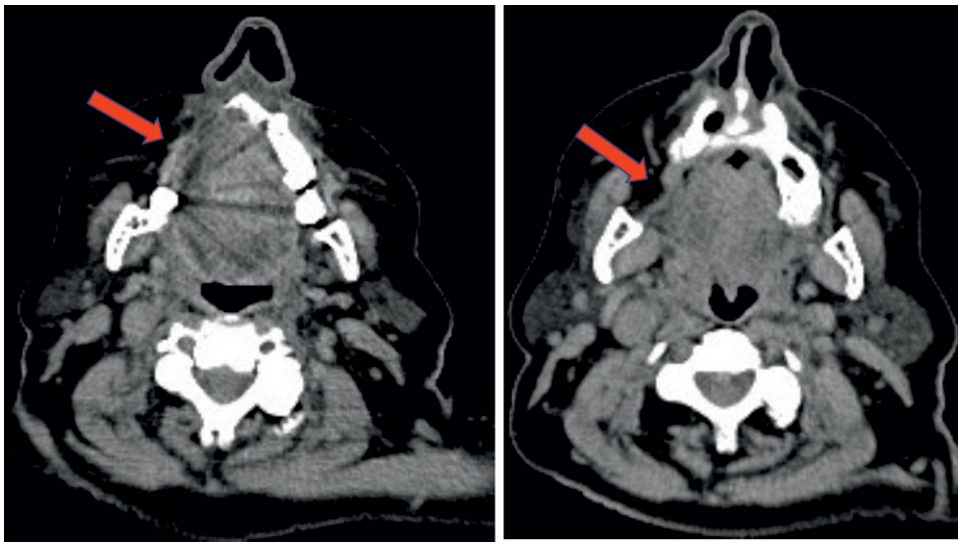

Figure 2. The current image of alveoli

regulator of bone mineralization) in which the central oxygen atom has been replaced by a carbon atom. Two additional side chains R1 and R2 are bound to the carbon atom. R1 is usually a hydroxyl group. Depending on the composition of R2, bisphosphonates can be divided into two major groups:

1. Simple (non-nitrogenous) bisphosphonates — without nitrogen in the R2 composition.
2. Nitrogenous bisphosphonates (aminobisphosphonates) — containing nitrogen in the R2 composition, including alendronate, zoledronate, pamidronate, risedronate, ibandronate [9, 10].

Bisphosphonates have a high affinity to hydroxyapatite crystals in bone, which they bind via two phosphate groups and the R1 side chain that together form the "bone hook" 11].

Aminobisphosphonates with a long side chain containing nitrogen cause disruption of the osteoclast intracellular signalling system, which results in the inhibition of the metabolic activity of mature osteoclasts. The clinical effect of aminobisphosphonates is inhibition of bone resorption [9].

Approximately 60% of the administered dose is deposited in areas of active bone mineralization, and its half-life is approximately 10 years. The non-deposited fraction is excreted by the kidneys [8]. The aetiology of BRONJ is unclear. It is caused by the exposure of maxillary or mandibular bone due to an interruption in

the continuity of the mucosa, e.g., during dental procedures or as a sequela of denture-related sores [10].

Because of the constantly applied pressures associated with chewing, the mandible and the maxilla are characterized by a greater bone turnover than other skeletal regions (e.g., 10–20 times greater than in the ilium) [12]. This intensity of bone turnover is necessary to repair microfractures arising during chewing. Concurrently, it requires a higher degree of vascularization, which is crucial in bisphosphonate therapy, allowing a higher concentration of these drugs in the bone tissue in that area [13]. The oral mucosa is thin and susceptible to damage, which facilitates bacterial infection of the exposed bone [14, 15].

Maxillary BRONJ leads to the formation of osteonecrotic areas. The disease presents similarity to radiation therapy-induced osteonecrosis, starting as aseptic necrosis of bone. Aseptic necrosis is usually caused by insufficient blood supply [16, 17].

In recent years, it was noted that similar lesions can occur in patients after treatment with denosumab (anti-RANKL IgG2) and bevacizumab (anti-VEGF antibody reducing tumour vasculature) [18], regardless of previous bisphosphonate therapy. There is also a number of factors that increase the risk of BRONJ. Among them, in the course of multiple myeloma, a likely genetic factor can be distinguished — polymorphism of the CYP2C8 gene that is linked to the arachidonic acid cycle and is a regulator of vascularization, which in the mandible could lead to poorer vascularity and increased risk of BRONJ (up to 12.5 times) [19].

Other BRONJ risk factors include glucocorticosteroids (chronic therapy adversely affects bone metabolism, weakening osteoblast differentiation and function). Furthermore, the immunosuppressive and antiangiogenic effect of glucocorticoids can play a major role in the development of necrosis [20–22].

The frequency of BRONJ increases from 1.5% (in patients treated for 4–12 months) to 7.7% (in patients treated for 37–48 months) [23].

The risk of this disease can also be increased by [24]: High alcohol consumption, smoking, anemia, chemotherapy, diabetes mellitus, obesity, renal failure, rheumatoid arthritis, immunosuppression, older age (risk increases by 9% per decade of life), female gender (maxillary BRONJ is 8 times more frequent in women than in men).

Local factors include [20] anatomical structures involving compact bone covered by a thin layer of mucosa, such as bony prominences or tubercles, periodontal diseases, including spontaneous tooth loss, surgical interventions related to the interruption in the continuity of oral mucosa, such as tooth extractions, periodontal treatments (scaling, curettage), placement of dental implants, endodontic therapy (when the tool is moved beyond the tip of the tooth root), misfitted dentures, poor oral hygiene.

The disease is usually diagnosed clinically. As a result of the lack of healing, areas of osteonecrosis occur and detach from the surrounding areas of purulent inflammation. The process is accompanied by pain, numbness, soft tissue oedema, hyperesthesia, tooth loosening, suppuration, and intra- and extraoral fistulas. Although these symptoms can develop spontaneously, BRONJ is much more frequent after surgical interventions on alveolar processes involving an interruption in the continuity of the mucosa and periodontium [8].

In some cases, necrosis develops asymptomatically and is clinically undetectable, with the patient being unaware of the disease for weeks or months. The first symptoms preceding clinically developed necrosis can include pain, mucosal ulceration, erythema and oedema, and tooth loosening [20]. In maxillary BRONJ, abscesses can reach the supra canine and buccal spaces [25]. In mandibular BRONJ, abscesses can be located in the submental, submandibular and sublingual spaces [26]. The difference in compactness between the maxilla and the mandible causes a different course of inflammation in these bones. In the maxilla, suppuration is manifested by a fistula, while in the mandible, suppuration spreads within the bone and rarely reaches its surface [27].

Three stages of progression of BRONJ have been distinguished [28].

Stage I — exposure of bone without oedema and erythema of the surrounding soft tissue, without radiological changes; pain can occur before bone exposure.

Stage II — primary or secondary inflammation of the soft tissue surrounding the exposed bone, pain, tooth loosening. Necrotic lesions in the radiological image can resemble periapical radiolucency, broadening of the periodontium or thickening of the alveolar lamina dura.

Stage II — primary or secondary inflammation of the soft tissue which is difficult to treat with oral or intravenous antibiotic therapy; the presence of extraoral fistulas. If the lesion affects the mandible, hypoesthesia of the lower lip can occur, and when the maxilla is affected, secondary maxillary sinusitis can develop. In the radiological image, a visible increase in radiolucency, pathological fractures of the mandible, necrotic areas, as well as osteitis-like and metastasis-like lesions can be found.

As has been reported in the literature, bacteriological tests can reveal the presence of *Staphylococcus epidermidis*, *Streptococcus salivarius*, *Morganella morganii*, *Prevotella intermedia* and *Prevotella oris*, as well as *Escherichia coli* — Gram-negative rods. In half of the cases, the exposed bone is colonized by *Actinomyces* strains [29].

Table 1. Methods of treatment of BRONJ [4]:

Stage of progression	Definition	Treatment
Stage I	— Loss of oral mucosa with bone exposure which can be preceded by pain — No radiological signs — No features of infection, edema or soft tissue erythema	Conservative (flushing of the oral cavity with, e.g., 0.12% chlorhexidine solution)
Stage II	— Loss of oral mucosa with bone exposure — Clinical features of infection — Soreness, tooth loosening — In X-ray, increased radiolucency, thickening of the alveolar lamina dura	Conservative (flushing of the oral cavity with, e.g., 0.12% chlorhexidine solution, and antibiotic/antifungal therapy)
Stage III	— Loss of oral mucosa with bone exposure — Clinical features of infection with pain — Signs, such as: fistula, pathological fracture, osteolysis, impaired sensation, sinusitis — In X-ray, increased radiolucency, necrotic areas, osteitis	— Surgical (resection of necrotic tissue or resection with vascularized bone grafting) — Antibiotic/antifungal therapy

The diagnosis of BRONJ is based on anamnesis, physical examination, clinical presentation and diagnostic imaging, usually pantomography or CT.

Treatment of patients with BRONJ is difficult. Treatment methods depending on the stage of progression and the extent of necrosis are shown in Table 1.

Conclusions

BRONJ is an increasingly observed complication following treatment procedures in the oral cavity of patients concurrently or previously receiving bisphosphonates. It is a very painful disease that is difficult to treat. Dental practitioners should be made more aware of the need for detailed anamnesis before conducting procedures that involve an interruption in the continuity of the oral mucosa. Previous treatment of oral problems in patients beginning bisphosphonate therapy, greater awareness of the need to inform the dentist of all medications taken currently and, in the past, as well as periodic checks and stricter hygiene regime might contribute to a reduction in the frequency of this complication.

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VIA MEDICA

CASE REPORT

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75-year-old man with lung cancer obscured by an implantable cardioverter--defibrillator — case report

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ABSTRACT

We present a case of a 75-year-old man, who underwent a scheduled implantable cardioverter-defibrillator reoperation and has had a lung cancer found in a chest X-ray taken after the procedure, that was completely obscured by the previous device.

Key words: Lung cancer, ICD, chest X-ray

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Introduction

Lung cancer is the most dominant form of cancer among men and the second one according to frequency among women in Poland [1]. In a group of implantable cardioverter-defibrillator (ICD) recipients, there are patients diagnosed with lung cancer but nationwide population-based cohort study in Denmark did not indicate a causal relationship between ICD implantation and cancer occurrence [2]. The aim of this case report was to point out not to forget about lung area hidden under the cardiac implantable electronic devices (CIEDs), as in the presented case the tumor was completely obscured by the previous device in prior chest X-ray.

Case report

A 75-year-old patient with coronary artery disease, hypertension, chronic left ventricular heart failure (New York Heart Association [NYHA] class II) and intermittent claudication was admitted to the Cardiology Department of University Hospital no 1 in Bydgoszcz for upgrade from ICD to cardiac resynchronization therapy defibrillator (CRT-D) with concomitant transvenous lead extraction (TLE).

The patient's medical history included an episode of ventricular fibrillation on 14th October 2009, coronary artery disease with a critical stenosis of right coronary artery on coronary angiography with subsequent unsuccessful, percutaneous coronary intervention. Echocardiographic examination at that time showed normal left ventricular ejection fraction (LVEF 55%). Patient was qualified for ICD implantation for secondary prevention of sudden cardiac death. On 28th October 2009 a single chamber ICD (Biotronik Lumax 300 VR-T) was implanted with a single coil Saint Jude Medical Durata lead. Defibrillation threshold test was successful at 18 J.

Between October 2009 and August 2015 the patient had multiple follow-up visits in the Outpatient Electrophysiology Clinic. In February 2012 the patient underwent electrical storm with multiple ICD discharges due to recurrent episodes of ventricular tachycardia and ventricular fibrillation. The only pathology identified as the cause was mild hypokalemia and the patient was started on amiodarone. Since September 2014 signs of electrode dysfunction were noted such as gradually growing ventricular pacing thresholds (from 1.2 V/1 ms in September 2014 up to 4.9 V/1 ms in July 2015) and increasing low-voltage circuit impedance (from 498 Ohm in September 2014 up to 2550 Ohm in July 2015), with normal and stable coil impedance and sensing

values. In July 2015 ICD battery elective replacement indication (ERI) status was reached. Due to ERI, signs of dysfunction of the high voltage lead and presence of clinical indications for cardiac resynchronization therapy (progression of heart failure — NYHA class II on admission, with concomitant left ventricular systolic dysfunction (left ventricular ejection fraction [LVEF] of 32%, and presence of left bundle branch block with QRS duration of 160 ms), the patient was qualified for replacement of the dysfunctional lead with concomitant upgrade from ICD to CRT-D.

The patient was admitted to the Cardiology Department on 27th August 2015. On admission he reported only moderate exertional dyspnoe with no other significant symptoms. Physical examination revealed enlarged axillary lymph nodes on the left side and lack of pulse on the popliteal and dorsal arteries on both sides (the patient had a history of intermittent claudication and was under supervision of vascular surgeon). Chest X-ray taken on hospital admission showed no abnormalities. The echocardiographic parameters were as follows: left ventricular ejection fraction: 32%, left ventricular end-diastolic diameter: 62 mm, left atrium diameter: 44 mm, interventricular septum diameter: 12mm. Low concentration of thyroid stimulating hormone (TSH) and slightly increased thyroid hormones prompted diagnosis of hyperthyroidism, probably secondary to amiodarone therapy and thiamazolum was added to the chronic treatment.

On 28th July 2015 the dysfunctional lead and the ICD can were successfully extracted and replaced with a Boston Scientific Origen CRT-D device with a Medtronic CapsureFix atrial lead, single coil Saint Jude Medical Durata high voltage lead and bipolar Saint Jude Medical Quickflex left ventricular lead. The surgery was uneventful.

As a routine post-procedural follow-up investigation, the standard posterior-anterior (PA) and lateral chest X-rays were taken (Figure 2. and 3.), which unexpectedly showed a round shape shadow in the upper pole of the left lung (area completely obscured by the previous device in pre-procedural X-ray — Figure 1.). Subsequent chest computed tomography revealed a pathological mass in the upper lobe of the left lung, sized 47x45x41 mm, with irregular, polycyclic borders, with contrast enhancement — suggestive for lung tumor. Abdominal ultrasound performed in search for possible metastatic foci showed no abnormalities. The patient was discharged from hospital and referred to a pulmonology department.

Subsequent telephone follow-up with the patient's family member revealed that the patient was qualified only for palliative therapy and eventually patient died in September 2016 due to respiratory failure.

Timeline of events

2009.10 — ICD implantation for secondary prevention of sudden cardiac death.
2012.02 — Electrical storm only with mild hypokalemia identified as the only reversible cause. Amiodaron added to therapy.
2014.09 — First signs of high voltage lead dysfunction.

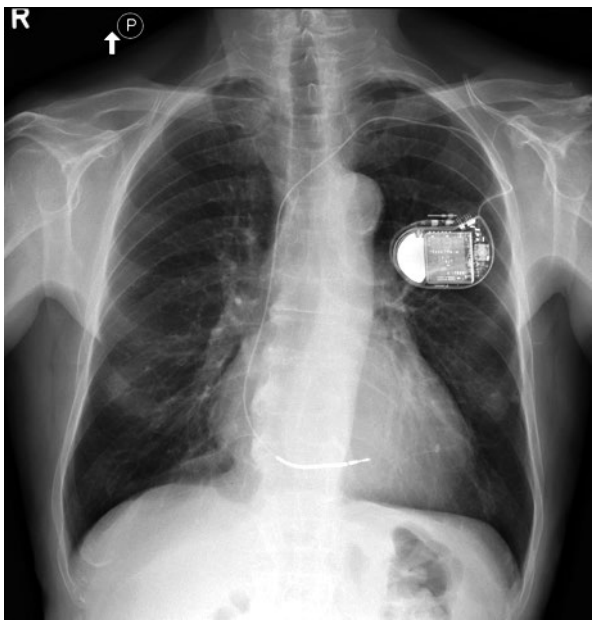

Figure 1. The posteroanterior chest X-ray taken before TLE procedure

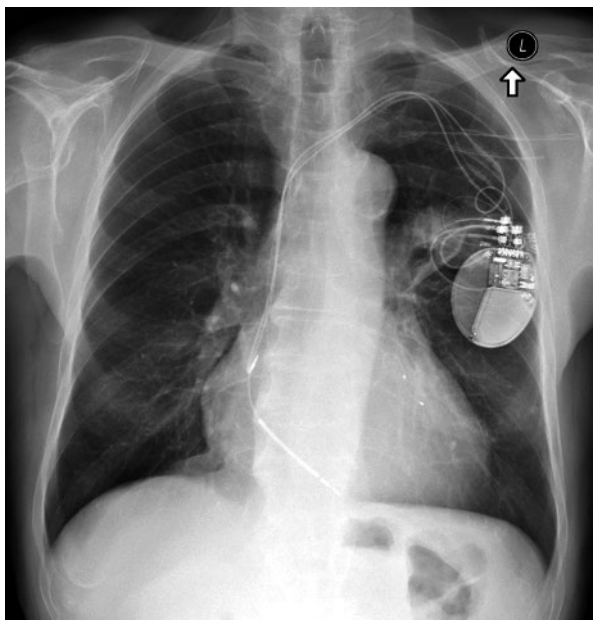

Figure 2. The posteroanterior chest X-ray taken after TLE procedure

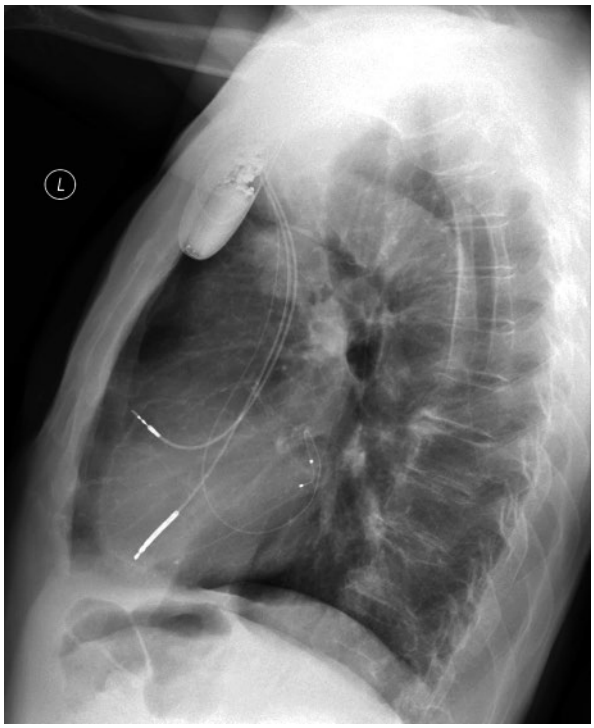

Figure 3. The lateral chest X-ray taken after TLE procedure

2015.04 — Follow-up visit in the Outpatient Electrophysiology Clinic: right ventricular (RV) pacing threshold: 2.8V/1.0 ms, an RV lead impedance: 1092 ohm.
2015.07 — Elective replacement indication status reached (low battery voltage).
2015.08.27 — Admission to the Cardiology Department. Normal admission chest X-ray.
2015.08.28 — Extraction of dysfunctional lead and ICD. Implantation of CRT-D.
2015.08.29 — Postoperative Chest X-ray displaying a round shadow (44x43x42 mm) in the upper lobe of the left lung cavity.
2015.09.03 — Abdominal ultrasound exam: no metastatic foci were found.
2015.09.04 — Chest CT scan: a pathological mass in the upper lobe of left lung- 47x45x41 mm, with irregular polycyclic and spicular outlines suggestive of lung tumor.
2015.09.04 — Discharge from hospital and referral to the Pulmonology Department.
2015.11 and 2016.06 — Follow-up visit in the Outpatient Electrophysiology Clinic: no arrhythmias.
2016.09 — Patient's death.

Discussion

The nationwide Danish population-based cohort study performed by Pedersen et al. did not indicate a causal relationship between ICD implantation and cancer, but the authors concluded that extended follow-up data are needed to elucidate risks for individual cancer types [2]. According to the literature, there have been cases of patients with CIEDs and lung cancer showing successful treatment of those patients with radiotherapy [3–4]. If radiotherapy is indicated or additional diagnostics is needed (i.e. lung biopsy) and it's not possible due to localization of CIED, there is always a possibility to relocate or remove the device. However, the radiotherapy is not the only possible treatment for lung cancer in the CIED recipients. In case of the interventional pulmonology procedures consultation with a physician involved in the CIED implantation is always required. It is usually necessary to change the parameters of the device for the time of surgery in order to prevent adverse events and more frequent follow-up visits in the Outpatient Electrophysiology Clinic in the course of radiotherapy are warranted.

The distinguishing feature of this case is an incidental diagnosis of lung cancer after relocation of the device during the exchange procedure. The pathological finding that was present prior to the surgery and could suggest a neoplasmatic process were the enlarged axillary lymph nodes. It was only the accidental change of the position of the device that allowed to visualize the previously invisible pulmonary field. In a case report presented by Bowers et al. two patients were also diagnosed with lung cancer directly posterior to a previously implanted ICD device, but in both cases, the new opacities were not completely overshadowed by the device [5]. One should be remembered that the field under the CIED may be not visible in the standard PA chest X-ray. This is important as the number of CIEDs recipient's is increasing and lung cancer is one of the most frequent cancers.

Learning points

The area under the CIED cannot be seen in the standard PA chest X-ray and this should be kept in mind. In order to visualize this lung area, there is a need for an additional side chest X-ray or a computed tomography (CT) scan.

In patients with CIEDs and symptoms suggestive of lung cancer, but normal results of the standard PA chest X-ray, extensive imaging methods should be applied.

Disclosure of interest: The authors declare that they have no conflict of interest

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