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The Editorial Board kindly informs that since 2014 *Nowiny Lekarskie* has been renamed to *Journal of Medical Science*.

The renaming was caused by using English as the language of publications and by a wide range of other organisational changes. They were necessary to follow dynamic transformations on the publishing market. The Editors also wanted to improve the factual and publishing standard of the journal. We wish to assure our readers that we will continue the good tradition of *Nowiny Lekarskie*.

You are welcome to publish your basic, medical and pharmaceutical science articles in *Journal of Medical Science*.

Ethical guidelines

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ORIGINAL PAPER

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Association between suicidal behavior and genes of serotonergic system confirmed in men with affective disorders

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ABSTRACT

Background. Suicidal behavior is a crucial clinical problem in many groups of psychiatric patients. It occurs most often in affective disorders, psychotic disorders, substance abuse/dependence and personality disorders. Although not all patients with these diagnoses present suicidal behavior, it is very important to find the most vulnerable subgroups. The extensive number of studies shows that suicidal behavior (completed and attempted suicide) is associated with changes in functioning of serotonergic system. Tryptophan hydroxylase (TPH) is rate-limiting enzyme in biosynthesis of serotonin. Serotonin transporter (5-HTT) is the main factor removing serotonin from the synaptic space. Genetic studies confirm that suicide behavior has a genetic component independently of major psychiatric disorders.

Aim. The aim of this study is to look for association between selected candidate genes and suicidal behavior in affective disorders.

Material and methods. In the study we included 597 patients meeting DSM-IV criteria for bipolar disorder or unipolar disorder and 563 healthy controls. Polymorphism of serotonin transporter gene 5-HTTLPR and single nucleotide polymorphisms – SNPs (rs1799913 and rs1800532) in tryptophan hydroxylase 1 (TPH1) gene were analyzed. We used in computation Statistica 8.0 package (STATSOFT, Poland) (tests: The two-tailed Pearson's chi-square ($\chi 2$) test and Fisher's exact test).

Results. Main positive findings are an association between TPH1 polymorphisms and bipolar disease type I (BPI) diagnosis in men and an association between TPH1 polymorphisms and suicidal attempts in male patients. In all group we did not find nor allelic neither genotypic associations of selected polymorphisms and diagnosis or suicide attempts.

Conclusions. Our findings partially confirm that serotonergic system plays a role in affective disorder and suicidal behavior, but the association needs further investigation.

Key words: suicide, affective disorder, serotonergic system genes.

Introduction

Suicidal behavior is a crucial clinical problem. Studies indicated that around 60% of suicide cases suffered from depression [1], but not all patients who suffer from mood disorder commit or attempt suicide. Around 50% of bipolar patients attempt suicide during their lifetime [2] and 20% commit suicide [3] Growing data show many clinical risk factors and indicate genetic predisposition both to psychiatric illness and suicide. Family, twin and adoption studies demonstrate that predisposition to suicide is transmitted

within families and is independent from psychiatric morbidity [4, 5].

Arguments on serotonergic hypothesis of depression (proposed by Lapin and Oxenkrug in 1969) have been investigated for 40 years. The large number of studies shows that suicidal behavior is associated with changes in functioning of serotonergic system [6–12].

Arango et al. performed quantitative autoradiographic study of prefrontal cortex in suicide victims compared to matched controls. They found that binding of serotonin 5-HT1A receptor (postsynaptic in cortex) was higher and serotonin transporter binding was lower in the suicide group. Serotonin transporter and 5-HT1A binding were negatively correlated in ventrolateral prefrontal cortex (VLPFC). That may suggest common regulatory factors [7]. Findings concerning binding of 5-HTT in depressive patients, who committed suicide, were similar: binding to 5-HTT was lower in the ventral PFC of suicides compared with nonsuicides [8]. Results of Stanley et al. were consistent: they revealed upregulated 5-HT2A receptors in ventral PFC of suicide victims [9].

Asberg et al. viewed the studies concerning levels of monoamines and its metabolites in suicidal persons. Authors revealed low levels of serotonin and 5-hydroxyindoleacetic acid (5-HIAA) in suicide victims [10].

Cooper et al. measured 5-HIAA in cerebrospinal fluid (CSF) from 30 schizophrenic patients and observed the group during 11 years. Patients who attempted suicide had lower levels of 5-HIAA in CSF than non attempters [11]. Moreover response to fenfluramine was reduced in patients with attempted suicide in lifetime history [12].

Association studies are focused on genes related to monoamines, especially serotonin. Serotonin is synthesized from tryptophan by tryptophan hydroxylase (TPH). TPH1 gene is located on chromosome 11p15.3-p14. It has two single nucleotide polymorphisms in intron 7: A779C and A218C. The meta-analysis performed by Bellivier et al. revealed an association between the TPH1 A218C polymorphism and suicidal behavior [13].

Abbar et al. analyzed 7 polymorphisms in TPH1 gene. 231 individuals who had attempted suicide and 281 controls were included in the study. Authors found (among others) association between 218A allele and violent suicidal behavior. The association was strongest in individuals, who had a history of major depression [14].

Serotonin is removed from synapse by serotonin transporter (5-HTT), sodium-dependent reuptake molecule. Its gene is located on chromosome 17q11.1–12. Due to ins/del of the 44bp fragment in serotonin transporter linked polymorphic region (5-HTTLPR), 5-HTT polymorphism has two alleles: long (L) and short (S). Homozygous LL genotype is associated with higher expression of 5-HTT [15]. It was hypothesized that S allele is associated with lower 5-HTT binding sites as it was found in suicide patients. Association between S allele and suicide was confirmed in several studies [16–18]. Other papers were not consistent: Du et al. observed higher frequency of L allele in suicide victims with depression [19]. In Mann's study authors did not find association between level of 5-HTT binding in prefrontal cortex and polymorphism of 5-HTT [8].

Aim

The aim of this study is to investigate an association between selected polymorphisms and suicide attempts exclusively in affective disorders. Based on previous published abundant literature [www. gmes.mcgill.ca, date of access: 04.2013] polymorphisms of TPH1 and 5-HTT were selected to the analysis.

Material and methods

Subjects

We included 597 patients (367 female, 230 male), aged 18–84 (mean = 47, SD ± 14) that met DSM-IV criteria for bipolar disorder BP (391 BPI, male n = 174; 104 BPII, male n = 33) or unipolar disorder UP (102 UP, male n = 23) living in Wielkopolska region of Poland. The diagnosis was established using SCID-I (Structured Clinical Interview for Axis I clinical disorders for DSM-IV). Attempted suicide was defined as self-destructive behavior with at least some intentions to end one's life [6]. In the interview we asked patients about the number and methods of attempted suicide. Among patients with BP, 197 persons had a history of suicide attempt(s), among UP – 28 persons had a history of suicide attempt(s). Suicide attempters were divided into two groups according to the suicide method: violent (n = 71; hanging, jumping from height or in front of vehicle, shotgun, exsanguination, sinking) or non violent [20,21]. We excluded patient who suffered single affective episode and patients, who committed suicide during clinical observation. Observed duration of disease in our group was: min: 1 year, max: 54 years (mean 15 years, SD 11). Time period between the onset of the disease and suicide attempt is illustrated at Figure 1.

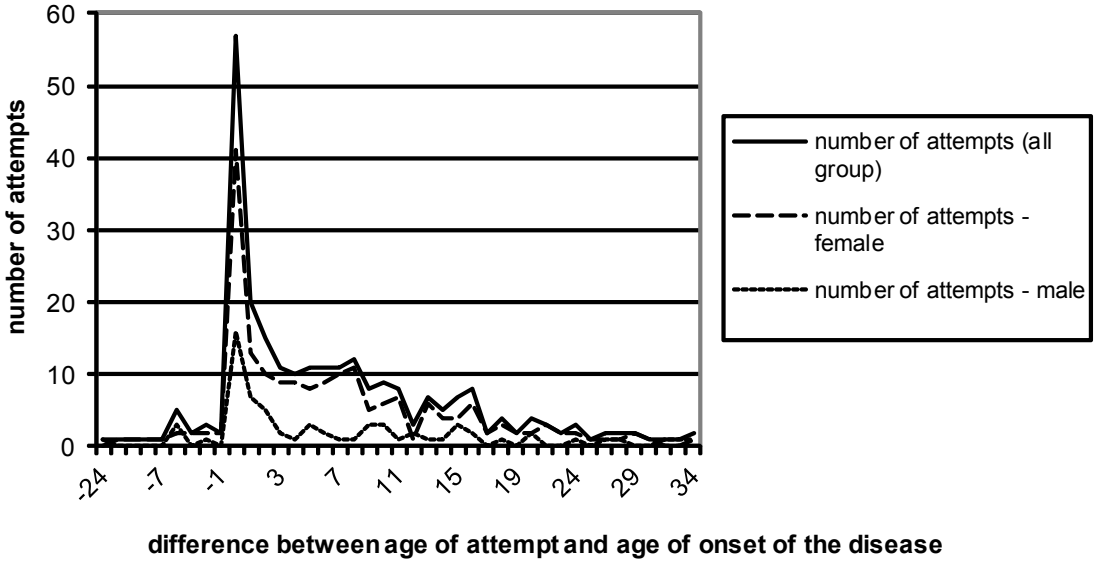

Figure 1. Time period between the onset of the disease and suicide attempt

The control group consisted of 563 healthy subjects (333 female, 230 male), aged 18–83 years (mean = 42 years, SD ± 13), from Wielkopolska region of Poland. Controls were recruited from blood donors, hospital staff, students and other volunteers.

The study was approved by the Ethics Committee, University of Medical Sciences in Poznan. All patients gave the written informed consent.

Genotyping

The selected intronic polymorphisms of TPH1 gene (rs1800532 and rs1799913) were genotyped using the TaqMan single-nucleotide polymorphism (SNP) allelic discrimination method with the ABI 7900HT system. In the serotonin transporter-linked promoter region (5-HTTLPR) we provided genotyping described by Stoltenberg et al. (2002) of the functional polymorphism of the serotonin transporter gene (5-HTT) [22].

The results of genotype and allele frequencies in patients and healthy controls with different TPH1 and 5-HTTLPR polymorphisms were analyzed using Statistica 8.0 package (STATSOFT, Poland) (tests: the two-tailed Pearson's chi-square ($\chi 2$) test for genotypic frequency analysis and Fisher's exact test for allelic frequency analysis). In all analyses $p < 0.05$ was considered statistically significant.

The discrepancy in the size of analyzed groups between "subjects" and tables are due to missing data in genotyping. The sample success rate for the genotyped polymorphisms was 97%. The reproducibility of the genotyping was 100%.

In genotypic analysis, we compared groups of all patients, BPI, BPII and UP to controls (Table 1) and we also compared suicide attempters and non attempters (accordingly to suggestions of Saetre et al. 2010 [23] (Table 2).

Results

The distributions of genotypes for all analyzed polymorphisms were in Hardy–Weinberg equilibrium in patients as well as in healthy controls ($p > 0.05$). Power analysis was performed using Quanto version 1.2.4. Power of association analysis obtained in our study was in range 0.05–0.26 (on the assumption that BP and UP prevalence is 2.1% and 17% respectively).

We found an association between TPH1 polymorphisms and BPI diagnosis in men. CA genotype of A779C polymorphism was significantly more frequent in male BPI patients than in controls. An A allele was significantly more frequent in male BPI patients ($p = 0.024$), and C allele was significantly more frequent in controls ($p = 0.029$). A218C CA genotype was significantly more frequent in male BPI patients than in controls. There is no significant allelic A218C association. We found no association between 5-HTTLPR polymorphism and diagnoses. Data on allelic frequency are not shown in tables.

The association between suicide attempts and genotype was investigated. We found an association between TPH1 A779C and A218C polymorphisms and suicidal attempts in men (Table 2), but it was not statis-

Table 1. Association between diagnosis of affective disorder and genotype

Polymorphism		TPH A779C rs1799913			p	TPH A218C rs1800532			p	5-HTTLPR			p
Genotype		CC	CA	AA		CC	CA	AA		SS	SL	LL	
BP+UP (n = 581)	T	33.27/32.35*	51.60/48.71	15.12/18.93	0.235	32.92/3.62	51.60/51.29	15.48/17.10	0.743	14.29/10.86	49.05/48.31	36.66/40.82	0.145
	F	34.68/30.63	48.84/50.63	16.47/18.75	0.489	34.88/30.53	48.55/53.57	16.57/16.20	0.427	14.04/9.55	47.47/47.77	38.48/42.68	0.170
	M	31.02/34.82	56.02/45.98	12.96/19.20	0.070	29.82/33.18	56.42/48.43	13.76/18.39	0.201	14.67/12.73	51.56/49.09	33.78/38.18	0.595
BPI(n = 356)	T	33.70/32.35	52.33/48.71	13.97/18.93	0.145	33.15/31.62	52.33/51.29	14.52/17.10	0.574	13.84/10.86	50.65/48.31	35.51/40.82	0.173
	F	39.51/30.63	45.85/50.63	14.63/18.75	0.094	39.90/30.53	45.32/53.27	14.78/16.20	0.085	14.55/9.55	47.42/47.77	38.03/42.68	0.181
	M	26.25/34.82	60.62/45.98	13.13/19.20	**0.017**	24.69/33.18	61.11/48.43	14.20/18.39	**0.048**	12.94/12.73	54.71/49.09	32.35/38.18	0.471
BPII (n = 102)	T	35.64/32.35	46.53/48.71	17.82/18.93	0.810	35.64/31.62	46.53/51.29	17.82/17.10	0.659	14.71/10.86	46.08/48.31	39.22/40.82	0.536
	F	27.94/30.63	48.53/50.63	23.53/18.75	0.658	27.94/30.53	48.53/53.27	23.53/16.20	0.352	10.14/9.55	49.28/47.77	50.58/42.68	0.948
	M	51.52/34.82	42.42/45.98	6.06/19.20	0.078	51.52/33.18	42.42/48.43	6.06/18.39	0.063	24.24/12.73	39.39/49.09	36.36/38/18	0.196
UP (n = 96)	T	29.17/32.35	54.17/48.71	16.67/18.93	0.614	29.17/31.62	54.17/51.29	16.67/17.10	0.862	15.63/10.86	45.83/48.31	38.54/40.82	0.406
	F	27.40/30.63	57.53/50.63	15.07/18.75	0.549	27.40/30.53	57.53/53.27	15.07/16.20	0.801	16.22/9.55	45.95/47.77	37.84/42.68	0.243
	M	34.78/34.82	43.48/45.98	21.74/19.20	0.952	34.78/33.18	43.48/48.43	21.74/18.39	0.883	13.64/12.73	45.45/49.09	40.91/38.18	0.948

BP – bipolar; UP – unipolar; BPI – bipolar type I; BPII – bipolar type II; T – total; M – males; F – females
** % of cases / % of controls*
bold numbers were used for statistically significant results; underlying was used for statistical trends (0.05<p < 0.06)

tically significant when we stratified suicide attempters by diagnosis. CA genotype of A779C polymorphism was significantly more frequent in suicide attempters (BP and UP together) than in controls, and AA genotype was significantly more frequent in controls. Similarly CA genotype of A218C polymorphism was significantly more frequent in suicide attempters (BP and UP together) than in controls, and AA genotype was significantly more frequent in controls. There was no significant allelic A779C and A218C association. We found, that for association between TPH1 A779C polymorphism and suicidal attempts in men, the dominant model is statistically significant (p = 0.0429). Models in other associations were not significant. We found no association between 5-HTTLPR polymorphism and suicidal behavior.

We also look for association of TPH1 A779C, TPH1 A218C polymorphisms, 5-HTTLPR and singular (n = 107) or multiple (n = 91) suicide attempters (Table 3). We found an association between TPH1 A779C polymorphism and singular suicidal attempts in men. CA genotype was significantly more frequent in this subgroup of suicide attempters than in controls. We observed an association between 5-HTTLPR and singular suicidal attempts in whole group of patients. SL genotype was significantly less frequent in singular suicide attempters than in controls, LL genotype was significantly more frequent in singular suicide attempters than in controls. There was no significant allelic association in these subgroups.

We did not observe any association between violent or non violent suicide attempts and analyzed polymorphisms (data not shown in tables).

Discussion

With respect to diagnosis, the results of association studies are still not concordant. We found association of BPI diagnosis with A218C and A779C polymorphisms (both CA genotype) of TPH1 in males and lack of association of these polymorphisms with UP. Meta-analysis performed by Chen et al. also shows, that A218C polymorphism of TPH1 is associated with bipolar (BP), but not unipolar (UP) diagnosis [24]. On the contrary in the study in Korean population authors found no association between TPH1 polymorphisms and diagnosis of bipolar disorder [25]. Negative findings were also reported by Craddock, Mendlewicz and Seretti [26–28].

We found no association of 5-HTTLPR and the diagnosis of affective disorder. Association of 5-HT-

Table 2. Association between suicide attempts and genotype in patients with affective disorders

Polymorphism		TPH A779C rs1799913			p	TPH A218C rs1800532			p	5-HTTLPR			p
Genotype		CC	CA	AA		CC	CA	AA		SS	SL	LL	
Suicide attempt(s) in lifetime history (n = 226) vs controls	T	29.44/32.35*	57.48/48.71*	13.08/18.93*	0.056	29.44/31.62*	57.48/51.29*	13.08/17.10*	0.235	13.64/10.86*	43.64/48.31*	42.73/40.82*	0.389
	F	30.77/30.63*	53.85/50.63*	15.38/18.75*	0.660	30.77/30.53*	53.85/53.27*	15.38/16.20*	0.976	13.70/9.55*	44.52/47.77*	41.78/42.68*	0.403
	M	26.76/34.82*	64.79/45.98*	8.45/19.20*	**0.013**	26.76/33.18*	64.79/48.43*	8.45/18.39*	**0.034**	13.51/12.73*	41.89/49.09*	44.59/38.18*	0.547
Suicide attempt(s) in lifetime history (n = 226) vs no suicide attempt in lifetime history (n = 371)	T	29.44/35.63*	57.48/47.99*	13.08/16.38*	0.091	29.44/35.06*	57.48/47.99*	13.08/16.95*	0.088	13.64/14.68*	43.64/52.35*	42.73/32.96*	0.057
	F	30.77/37.44*	53.85/45.32*	15.38/17.24*	0.286	30.77/37.81*	53.85/44.78*	15.38/17.41*	0.245	13.70/14.29*	44.52/49.52*	41.78/36.19*	0.556
	M	26.76/33.10*	64.79/51.72*	8.45/15.17*	0.155	26.76/31.29*	64.79/52.38*	8.45/16.33*	0.150	13.51/15.23*	41.89/56.29*	44.59/28.48*	0.052
Violent suicide attempt(s) in lifetime history (n = 65) vs controls	T	32.31/32.35*	58.46/48.71*	9.23/18.93*	0.125	32.31/31.62*	58.46/51.29*	9.23/17.10*	0.247	11.76/10.86*	42.65/48.31*	45.59/40.82*	0.676
	F	34.38/30.63*	56.25/50.63*	9.38/18.75*	0.419	34.38/30.53*	56.25/53.27*	9.38/16.20*	0.589	8.82/9.55*	55.88/47.77*	35.29/42.68*	0.660
	M	30.30/34.82*	60.61/45.98*	9.09/19.20*	0.212	30.30/33.18*	60.61/48.43*	9.09/18.39*	0.306	14.71/12.73*	29.41/49.09*	55.88/38.18*	0.090
Not violent suicide attempt(s) in lifetime history (n = 133) vs controls	T	28.57/32.35*	56.39/48.71*	15.04/18.93*	0.268	28.57/31.62*	56.39/51.29*	15.04/17.10*	0.570	13.97/10.86*	43.38/48.31*	42.65/40.82*	0.459
	F	29.41/30.63*	53.92/50.63*	16.67/18.75*	0.825	29.41/30.53*	53.92/53.27*	16.67/16.20*	0.976	14.56/9.55*	39.81/47.77*	45.63/42.68*	0.219
	M	25.81/34.82*	64.52/45.98*	9.68/19.20*	0.137	25.81/33.18*	64.52/48.43*	9.68/18.39*	0.218	12.12/12.73*	54.55/49.09*	33.33/38.18*	0.835
Suicide attempters (n = 226) vs non attempters (n = 371)	T	29.44/35.63**	57.48/47.99**	13.08/16.38**	0.091	29.44/35.06**	57.48/47.99**	13.08/16.95**	0.088	13.64/14.68**	43.64/52.35**	42.73/32.96**	0.056
	F	30.77/37.44**	53.85/45.32**	15.38/17.24**	0.286	30.77/37.81**	53.85/44.78**	15.38/17.41**	0.245	13.70/14.29**	44.52/49.52**	41.78/36.19**	0.556
	M	26.76/33.10**	64.79/51.72**	8.45/15.17**	0.155	26.76/31.29**	64.79/52.38**	8.45/16.33**	0.150	13.51/15.23**	41.89/56.29**	44.59/28.48**	0.052

BP – bipolar; UP – unipolar; BPI – bipolar type I; BPII – bipolar type II; T – total; M – males; F – females
** % of cases / % of controls*
*** % of attempters / % of non attempters*
bold numbers were used for statistically significant results; underlying was used for statistical trends (0.05 < p < 0.06)

Table 3. Association between singular/multiple suicide attempts and genotype in patients with affective disorders

Polymorphism		TPH A779C rs1799913			p	TPH A218C rs1800532			p	5-HTTLPR			p
Genotype		CC	CA	AA		CC	CA	AA		SS	SL	LL	
Patients with singular suicide attempt (n = 107) vs controls	T	27.10/32.35	57.94/48.71	14.95/18.93	0.222	27.10/31.62	57.94/51.29	14.95/17.10	0.456	14.41/10.86	34.23/48.31	51.35/40.82	**0.025**
	F	30.43/30.63	52.17/50.63	17.39/18.75	0.984	30.43/30.53	52.17/53.27	17.39/16.20	0.983	14.08/9.55	32.39/47.77	53.52/42.67	0.059
	M	21.05/34.82	68.42/45.98	10.53/19.20	0.043	21.05/33.18	68.42/48.43	10.53/18.38	0.091	15.00/12.73	37.50/49.09	47.50/38.18	0.397
Patients with multiple suicide attempts (n = 91) vs controls	T	32.97/32.35	56.04/48.71	10.99/18.93	0.157	32.97/31.62	56.04/51.29	10.99/17.10	0.353	12.22/10.86	52.22/48.31	35.56/40.82	0.605
	F	30.77/30.63	56.92/50.63	12.31/18.75	0.456	30.77/30.53	56.92/53.27	12.31/16.20	0.775	12.50/9.55	54.69/47.77	32.81/42.68	0.316
	M	38.46/34.82	53.85/45.98	7.69/19.20	0.363	38.46/33.18	53.85/48.43	7.69/18.39	0.452	11.54/12.73	46.15/49.09	42.31/38.18	0.957

bold numbers were used for statistically significant results; underlying was used for statistical trends (0.05<p < 0.06)

TLPR with BP, but not with depression, were shown in meta-analysis performed by Anguelova et al. No polymorphism of TPH1 was considered in this study [29]. Brezo et al. found that TPH1 variation was relevant in the diathesis for suicide attempts and 5-HTTLPR in mood disorders [30].

We found association between TPH1 genotypes and suicidal attempts in men. In both investigated loci CA genotype was more frequent in suicide attempters, and AA genotype was more frequent in controls. The result is consistent with recent meta-analysis. Clayden et al. concluded (although it may not be so simple), that A218C polymorphism of TPH was associated only with suicide attempts, not with completed suicide [31]. Indeed several studies concerning suicide completers (regardless psychiatric morbidity) gave not consistent results (n = 247, only males, association with GG genotype in A218C SNP) [32]; (n = 160, males, only violent suicides, no allelic or genotypic association) [33].

In study performed by Tsai et al. 151 affective patients and 200 controls were included. An association between TPH A218C polymorphism and suicidal behaviors was found in depressive patients, but not in bipolar. The A allele and AA genotype were associated with suicide [34]. In recent study Galfalvy et al. genotyped 343 subjects presenting a Major Depressive Episode. The group was ethnically heterogeneous (Caucasian, African-American, Hispanic). Clinical factors and CSF-HIAA (5-hydroxyindoloacetic acid), HVA (homovanillic acid) and MHPG (3-methoxy-4-hydroxyphenylglicol) levels were also explored. Subjects have been monitored for suicide attempts for one year. AA genotype of A218C polymorphism was associated with history of high-lethality suicide attempts and predicted suicide attempts during the 1 year follow-up [35]. Association found in our group was different (CA genotype of A218C was significantly more frequent in suicide attempters than in controls).

In 1997 Mann et al. performed a study on group of 51 inpatients with major depression. 29 of them attempted suicide. Patients who had attempted suicide and patients who had not were comparable in severity of depression (rated with the 17-item Hamilton Depression Rating Scale). Persons who attempted suicide (N = 29), made an average of 2.2 attempts during their lifetime. Authors found an association between allele A and genotype AA of A779C polymorphism and suicide [36]. In our study comparing suicide attempters (n = 226) and controls (n = 544) we obtained discordant result. AA genotype of A779C was significantly more frequent in controls than in suicide attempters.

We found no statistically significant association comparing group with (n = 226) and without (n = 371) suicide attempts.

In numerous studies no association between alleles and/or genotypes of TPH1 and suicide behavior was found. Negative results authors reported: taking into consideration association with suicide ideation [19]; in groups of suicide completers [37–40]; in groups of suicide attempters [23, 41–47].

We also did not find association between 5-HTTLPR and suicide attempts which was confirmed by many studies. In affective patients such results reported also de Luca et al. 2005, 2008 [48,49], Zalsman et al. 2006 [50], Mendlewicz et al. 2004 [26], Bellivier et al. 2000 (genotypic not significant) [17] Ho et al. 2000 [51], Ohara et al.1998 [52]. Among the papers mentioned above the study of Mendlewicz et al. stands out because of a very large investigated group (572 BP, 539 UP, 104 suicide attempters and 821 controls) and selection regarding diagnosis. The study was performed in 8 European centers.

In 1999 Du et al. reported association between L allele of 5-HTTLPR and suicide. Our results obtained in subgroup of patients, who attempted singular suicide attempt (n = 107), are consistent with it (although Du et al. considered group of only 24 suicide completers with well documented diagnosis of depression [19]). Similar results reported in 2000 Faludi et al. (n = 32) [37]. Contrary, Bondy et al. (2000) found that suicide victims were significantly more often carriers of SS or SL genotypes than controls. This group differs from our patients, because they were suicide completers with unknown psychiatric diagnosis [53]. Gorwood et al. reported association between SS or SL genotype in alcoholic patients with history of suicide attempts. Authors analyzed 110 alcohol-dependent males and 61 unaffected blood donors. It is possible that different diagnoses are important for effect of serotonin transporter polymorphism [54]. Analyze carried out by Caspi et al. showed that stressful life events predicted suicide ideation or attempt among individuals carrying an S allele but not among LL homozygotes. It suggests the importance of gene and environment interaction and its influence to depression course [55]. Coventry et al. tried to explore interactions of stressful life events and genotype in depressive patients. Self-reports of depression and an increase in depression/suicidality were significantly associated with prior personal events. They were not significantly associated with any of the genotype main effects (5-HTTLPR, 5-HTTLPR + rs25531) or interactions (stress x genotype) [56].

Concerning violent and non violent suicide attempts, we did not find association with investigated polymorphisms in these subgroups of patients. Bellivier et al. compared 194 unipolar and 43 bipolar patients (99 suicide attempters) and 187 blood donors as control group. They found no genotypic association with 5-HTTLPR and no allelic association in all suicidal groups. Authors found allelic association between S allele and subgroup of violent suicide attempters. The more severe was the phenotype (i.e. control subjects, no suicidal behavior, non violent attempt, and violent suicide attempt), the higher was the frequency of the S allele [17]. Neves et al. twice reported allelic association between S allele and suicidal and violent suicidal behavior. Firstly on group of 167 BP patients (2008) [57], and then on 198 BP patients (2010) [58]. In both papers the investigated group had different ethnicity than our group and number of suicide attempters was lower. Meta- analysis carried out by Li and He in 2007 supports the hypothesis of role of 5-HTT in the pathogenesis of suicidal behavior [59].

Our findings confirm that serotonergic system may play a role in affective disorder and suicidal behavior, but this association needs further investigation. Due to lack of statistically significant association in allelic analysis the interpretation of genotypic results is more difficult. We may suppose that the influence of polymorphic genes of serotonin neurotransmission may partly vary in male and female. Investigation performed by Perroud et al. in 2010 supports it. Authors found that females had higher TPH1 and 5-HTT transcripts levels than males. Whereas these expression levels did not differ between suicide completers and controls [60].

The results of association studies are still not concordant. Findings differ probably because of ethnicity differences, varied analyzed diagnostic groups or not homogeneous investigated groups. Investigated groups may not be representative for whole population of patients (some persons who suffer for personality disorders may refuse participation in studies) and healthy controls. One can suppose that influence of single polymorphism is so little that it is sometimes hardly noticeable because of the influence of other factors (like life events or personality traits). These elements are especially important in so complex phenomenon as suicidal behavior. Several authors suggest that genes act through intermediate phenotypes, that may be separate from psychiatric diagnosis [61].

Strategy using endophenothypes may be more useful in genetic studies than pure, but complex in signs and symptoms, diagnosis [62].

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Microspectral laser analysis of selected dental implants

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ABSTRACT

Aim. In the paper the microspectral analysis of selected dental implants was presented.
Material and methods. The following implants were analysed: Osteoplant, Keystone Dental, Mozo Grau, Alpha Bio, Sky, and Implant Direct. The probes of each implant were analysed in the laser spectrophotometer.
Results. The result of the study was: Keystone Dental, Mozo Grau, Alpha Bio and Implant Direct titanium implants were shown to include aluminium and vanadium components while the Osteoplant and Sky implants to be made of pure titanium.

Key words: dental implants, microspectral laser analysis, titanium purity.

Introduction

Dental implants made of titanium are used to treat missing teeth in more and more difficult bony conditions. Therefore, it is required to improve their mechanical strength during their manufacture. It is achieved by modifying titanium alloys composition through the application of admixtures or additions.

Because of its properties titanium and its alloys have been used in medicine for many years. This metal was discovered by William Gregor in 1791 and, independently, by Martin Heinrich Klaproth in 1795 [5]. However, it was only in the 1950s that it started to be used commercially. Titanium is obtained from titanium ores: rutile, ilmenite, titanomagnetite, or a mixture of these ores. Main titanium alloy additions are: Al, Sn, Mo, V, Mn, Fe, Cr. Alloy elements, being dissolved in titanium, increase its strength with Fe, Cr and Al producing the greatest hardening effect. The additions affect the temperature of allotropic transformation, too. Some of them, like Cr, Mn, Fe, Al show limited solubility and form intermetalic compounds enabling alloy precipitation hardening. Still, this effect is slight. Depending on their chemical composition titanium alloys may be of a monophase α, monophase β, or diphase $\alpha + \beta$ structure [8]. Monophase α alloys show very good resistance to corrosion and oxidation, good weldability, resistance to brittle cracking, and creep strength, better than presented by the β alloys. Still, the α alloys are characterised by worse strength and deformability than the β alloys. The diphase $\alpha + \beta$ structure alloys combine the advantages of the α and β alloys. The most commonly used alloy addition in the α and β alloys to stabilise and enhance the α phase is Al, which also increases its thermal stability together with decreasing the alloy density. The β-isometric elements (V, Mo, Nb, Ta) cause an increase in the plasticity of the β phase. In general, the $\alpha + \beta$ alloys show good strenght, plasticity and corrosion resistance. With an increase of the β-phase component mechanical properties of the $\alpha + \beta$ alloy improve, reaching its maximum in a structure composed of 50% of the α phase and 50% of the β phase. The most commonly used Ti6A14V alloy belongs to this group. It displays an effective combination of strength, resistance to brittle cracking and fatigue strength [2]. This alloy is applied in the production of numerous elements in aviation and cosmic industry as well as in the production of surgical implants. In the construction of

machines and devices there is already a wide spectrum of the application of this alloy, especially in the parts exposed to a dynamic load. Titanium alloys such as Titanium Grade 5ELI, which are used to manufacture surgical implants, are resistant to corrosion, bio-compatible and bio-connective. These features result in the alloys to be practically adopted by the human body what enables the cells to connect to an implant [1, 6]. There are reports in literature showing aluminium and vanadium, the Ti6A14V alloy components, to be toxic. Therefore there are attempts to substitute vanadium with niobium (Ti6A17Nb) [3, 9].

One of the features likely to produce a negative effect on the tissues adjacent to an implant, and the whole body too, is release of trace amounts of metals which are the implants components. Therefore, the authors have undertaken an effort to independently and objectively determine the presence of impurities and additions of metals in dental implants.

Aim

The aim of the paper is to test the presence of metalic impurities found in selected titanium implants, using microspectral laser analysis.

Material and methods

Six different titanium implants were examined: Osteoplant, Keystone Dental, Mozo-Grau, Alpha Bio, Sky, Implant Direct (Figure 1).

A laser microprobe was used in the study. A laser microprobe includes a neodymium laser with a supply system, an optic microscope for work in a reflected light, an impulse excitation source, and a spectrograph. A laser impulse is to evaporate a microscopic volume of the studied sample, which is later excited with an impulse of a spark discharge. Evaporation and excitation is a one-stage process.

Both the observation of the studied sample field and focusing of a laser beam is done with the use of a special microscope objective lens. The lens and mirror assembly allows a marked distance between the focus and the objective lens surface, maintaining a very short focal length. Such a construction of an optical system ensures a big magnification of the microscope (500x) and its protection against possible damage done by the evaporated material from the studied sample. Laser head is equipped in another objective lens of a bigger focal length designed to fix additional electrodes in the laser beam axis, to control a distance between them, and to observe bigger fragments of the studied sample (50x magnification). The microscope optical system is secured against premature triggering of the laser impulse during the observation of sample surface. The profile and dimensions of the crater created on the examined material depend of the laser working conditions, its output and power density as well as on the thermal properties of a sample material [4, 7] (Figure 2).

Figure 1. An Osteoplant implant prepared for the examination

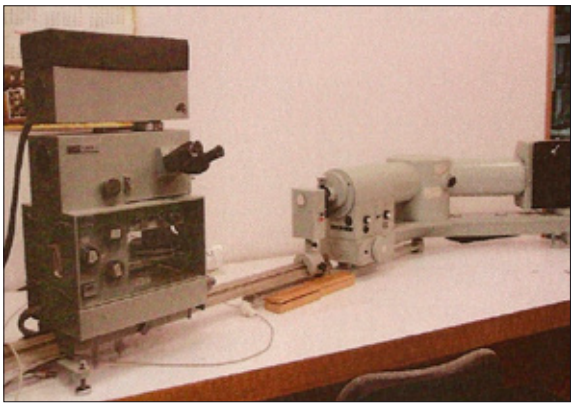

Figure 2. A laser microprobe. On the left – a laser microanalyser head, on the right – a Q-24 spectrograph for spectrum registration

Result and discussion

The results of the microspectral laser analysis of the selected dental implants are presented on figures (Figures 3–6). On some pictures aluminium and vanadium lines are visible indicating the presence of these metals. On some others, there are no metalic traces except for titanium. Figure 4 shows a fragment of the examined Keystone Dental implant spectrum where the lines corresponding with the range of aluminium can be seen (indicated with the blue arrows).

To sum up, laser analysis showed that the titanium implants by Keystone Dental, Mozo Grau, Alpha Bio and Implant Direct include aluminium and vanadium

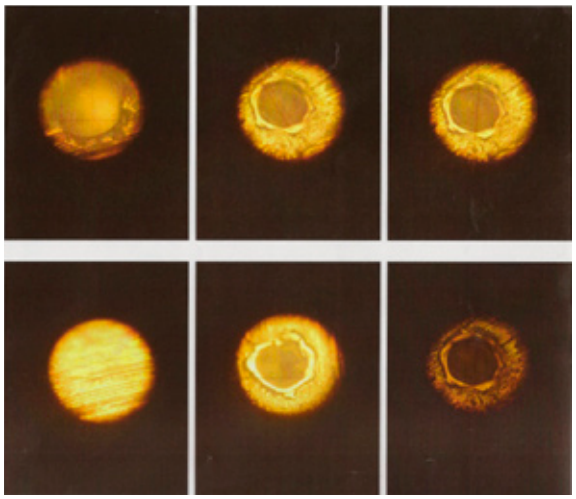

Figure 3. Pictures of the analysed sample surface following the laser impulse. Crater diameters range from 50 to 70 μm

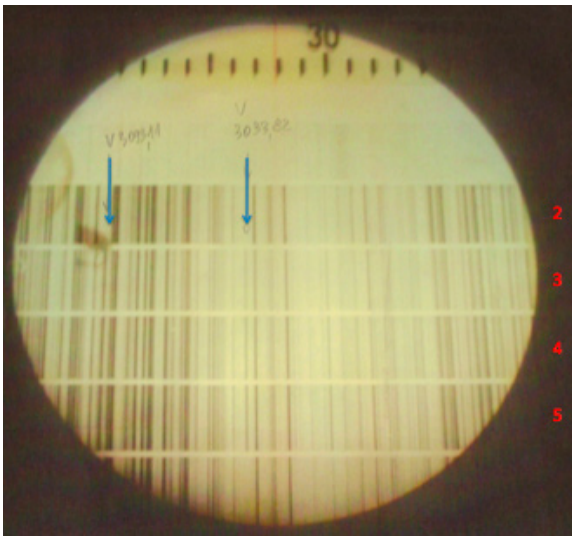

Figure 5. A spectrum of the studied Keystone Dental implant (fragments 3, 4 and 5) showing the lines characteristic of vanadium. Fragment 2 corresponds with the standard

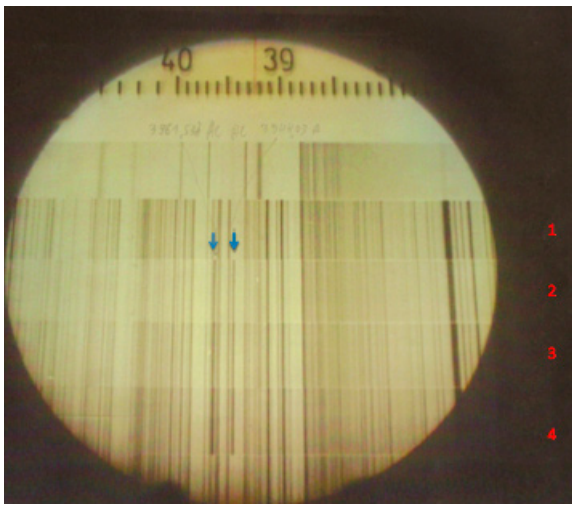

Figure 4. A plate with a fragment of a Keystone Dental implant spectrum (fragment 2, 3, and 4). The blue arrows pointing to the lines indicate the presence of aluminium in the studied material. Fragment 1 shows no line as it corresponds with the titanium standard. Fragments 2, 3 and 4 include aluminium found in various places in the studied implant

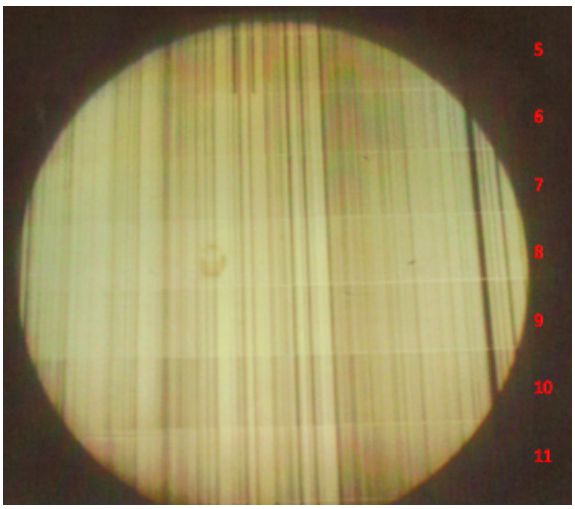

Figure 6. A fragment of a plate presenting the studied Osteoplant implant with no lines indicating the presence of aluminium (fragments 6, 7, 8, 9, 10 and 11). Fragment 5 corresponds with the Keystone Dental implant and a line indicating aluminium is visible on it

additions and therefore they are titanium-aluminium-vanadium alloys while the implants by Osteoplant and Sky do not include additions of other metals are are pure titanium products.

The basic aim of spectral analysis is determination of the qualitative and quantitative composition of the examined materials basing on their emissions spectra. In the qualitative studies the appearance of special lines in the spectrum indicates the presence of a given element in the source of excitation. The qualitative laser spectral microanalysis does not pose many difficulties. Still, it is essential to observe procedure rules to ensure that the obtained spectrum comes from the studied sample and not from the electrode material on which some evaporated material from the previous sample is likely to deposit.

On the other hand, the quantitative study may cause bigger problems. Here, it is essential to maintain identical parameters of a laser impulse when photographing the spectra of the studied sample and the standards. An additional difficulty is a choice of appropriate internal standards which must have homo-

geneous composition as the laser micoanalysis is of a local character. If it is only possible, the whole analytic process should be done basing on one impulse of a laser because numerous experiments displayed poor correlation between the line intensity and mass of the evaporated sample. The lack of correlation may be also due to composition nature of the sample (its heterogeneity) as well as to explosive character of evaporation during which, apart from volatile elements, liquid and solid elements may be expelled.

Laser microanalysis shows an advantage over other methods of analysis with regard to:

- performing the analysis without possible damage to the structure of the studied object. The crater diameter formed in a studied material by one laser impulse is about 100 μm, the loss in its mass is 10^{-6}–10^{-7} g. It allows to detect the components as small as some parts per million (ppm)
- performing local analysis, i.e. determining the chemical composition of a studied heterogeneous sample in its concrete place. The advantage of this method is that it does not require special preparation of a sample. Laser microanalysis allows specification of the type of inclusions, defects of materials and impurities in various substances.

Metals harmfulness and their influence on the human body cannot be ignored. Studies which consist of laser microspectral analysis ensure precise determination of trace amounts of metals in the studied samples. Such detailed analysis makes a starting point for further research on real release of trace elements from these implants since their presence does not imply their release. Still, confirmation of their presence is indispensable for precise evaluation of a potential risk their release may pose on the environment. Dental implants manufacturers are constantly improving the composition and structure of implants surface. However, they do not reveal the implants structure and composition and the reason they give is to keep it secret from their competitors on the market.

Conclusions

1. Titanium implants by Keystone Dental, Mozo Grau, Alpha Bio and Implant Direct are titanium-aluminium-vanadium alloys.
2. Implants by Osteoplant and Sky are made of pure titanium.

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Adoptive transfer of tumor specific T cells from allogeneic donors is feasible, effective and safe alternative to autologous T cell based tumor immunotherapy

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ABSTRACT

Donor lymphocyte infusion is used to increase the graft versus tumor (GVT) effect after allogeneic hematopoietic cell transplant. The limited spectrum of activity and high risk of graft versus host disease (GVHD) remain major limitations of this approach. The finding of new cell populations for adoptive immunotherapy, with the ability to separate GVT from GVHD, would be useful. In the present manuscript, we tested in mouse model the use of allogeneic MHC partially matched effector cells for adoptive T cell immunotherapy of cancer. We sought to maximize graft-versus-tumor effect while minimizing GVHD using tumor-specific allogeneic effector T cells rather than open-repertoire T cells. A F1 hybrid (Balb/c x C57BL/6) -MethA-EGFP–bearing mice received a preparative regimen of nonmyeloablating cyclophosphamide lymphodepletion followed by adoptive transfer of bulk Balb/c derived allogeneic T cells specific for the MethA-EGFP tumor cells. Adoptively transferred allogeneic tumor-specific T lymphocytes prevented tumor formation without graft versus host disease – like symptoms. We found that the risk of GVHD was low even with high number of transferred tumor-specific T cells. These data indicate that the use of tumor-specific allogeneic T cells is feasible, effective and safe alternative to autologous T cell based tumor immunotherapy.

Key words: adoptive cell transfer, tumor immunotherapy, cancer, GVHD, allogeneic T cells, murine model.

Introduction

Allogeneic bone marrow or stem cell transplantation can be considered the most successful method of adoptive immunotherapy of malignances. Its success appears to be related in part to the graft-versus-tumor (GVT) potential of the donor graft. Donor T-cells may recognize leukemic specific or recipient specific alloantigens and eliminate residual malignant cells (graft versus leukemia). This T-cell reaction can be harnessed in some cases to treat patients who relapse after allogeneic stem cell transplantation with the use of donor leukocyte infusions (DLIs). Unfortunately, the effects of GVT have clear disease specificity. Despite its therapeutic potential in hematologic malignancies most solid tumors have proven to be resistant to this therapy [1–8]. Thus, GVT induction in solid tumors require a better understanding of the important target antigens and effector cells, as well as the development of methods that enhance GVT reactivity without excessive GVHD.

A factor that might be important in GVT is donor T cell tumor specificity. In DLI vast majority of T cells do not have any specific tumor reactivity, have unknown

antigen specificity and possess an intrinsic danger of GVHD induction. The absence of defined antigen specificity of such T cells makes them difficult to reproducibly create a therapeutic window between GVT and GVHD. In contrary to DLI tumor infiltrating lymphocytes (TIL) may contain T cells with antitumor activity. Indeed, adoptive cell transfer (ACT) of TIL amplified *in vitro*, in association with lymphodepleting chemotherapy and IL-2, has been shown to mediate regression of large established tumors [1–2]. Despite its clinical efficacy this approach is still experimental treatment for patients with metastatic melanoma. Its wide use is limited by the fact that cells must be obtained through a surgical procedure, followed by the process of ex vivo expansion, which is difficult, labor intensive and it is not always feasible to yield T cells in amount sufficient for clinical purposes [3]. Allogeneic donor tumor-specific T–cells partially matched for recipient MHC antigens could provide an alternative to autologous TIL therapy.

The use of allogeneic antigen specific T cell therapy has been already reported. It was shown that allogeneic TILs sharing an MHC restriction element for a common tumor antigen can be used to successfully treat established metastases in the nonallosensitized host [7]. Additional preclinical results used an experimental model of melanoma treated with transgenic antigen specific allogeneic major histocompatibility complex (MHC)–mismatched T-cell. These adoptively transferred allogeneic tumor-specific T lymphocytes persisted at detectable levels for several weeks and mediated significant regression of large, vascularized tumors [9]. Together, these results encourage the implementation of further studies assessing the safety and clinical efficacy of tumor specific T cells administered to cancer patients bearing antigen-expressing tumors. The use of this strategy raises two major concerns: the survival of the effectors and the risk of development of lethal graft versus host disease. Whereas the persistence of transferred cells might be crucial for a favorable clinical outcome [10–12], the use of allogeneic T cells in immunocompromised host have a high risk of deleterious graft versus host disease which is a major, potentially life threatening complication. The most important factor predicting the risk of GVHD is MHC disparity between the donor and recipient. Therefore in cases with only partial MHC matching the probability of GVHD after ACT might be potentially very high. Although it was found that the risk of GVHD was low when allogeneic tumor-specific T cells were transferred however this study was based on transgenic antigen specific T cells where the majority of transferred cells had well defined antigen specificity. In clinical situation allogeneic tumor reactive T cells may have unknown specificity and possess a potential danger of inducing lethal GVHD. In the present paper, we sought to explore the feasibility of using tumor-specific allogeneic T cells in the treatment of the experimental mouse sarcoma MethA, after a preparative lymphodepletion regimen with cyclophosphamide (CPA). We tried to simulate clinical condition with in vivo antigen T cell priming followed by in vitro expansion and semiallogeneic adoptive transfer. We studied the ability of allogeneic cells to reject tumor cells and explored the risk of inducing GVHD-like reactions when tumor primed allogeneic T cells are used as a cell source.

Material and methods

Mice and tumor lines

All mice used in these experiments were housed at the Animal Facility of University of Medical Sciences, Poznan. Female Balb/c (H-$2^{d/d}$) and F1 hybrid Balb/c x C57BL/6 (H-$2^{b/d}$) mice were used. Female F1 hybrid Balb/c x C57BL/6 and Balb/c wild-type were used as recipients in ACT experiments. Balb/c mice (H-$2^{d/d}$) were used as a source of donor cells. All mice were purchased Mossakowski Medical Research Centre Polish Academy of Sciences, Warsaw, Poland.

The MethA-EGFP tumor cell line is derived from a chemically induced sarcoma in Balb/c mouse (H-2^{d}) and expresses the EGFP antigen [16, 17] MethA-EGFP cells were maintained in culture at 37°C in 5% CO_2 in CMM. Experiments were conducted with the approval of the University of Medical Science Animal Use and Care Committee according to national regulations.

In vitro expansion of anti-MethA-EGFP specific T cells

Anti-MethA-EGFP T cells were isolated from spleens of Balb/c donor mice immunized twice with 10^6 irradiated (10Gy) MethA-EGFP cells in weekly intervals. After 8–10 days splenocytes were isolated and cultured in the presence of briostatin (5 nM), ionomycin (1 μM) and IL-2 (80 U/ml) in X-Vivo 10 medium for 16 hours. Then, bulk T cell cultures were plated at 10^6 cells/mL in X-Vivo 10 medium with IL-7 (20 ng/ml) and IL-15 (20 ng/ml). After next 24 hours the medium was supplemented with IL-2 (20 U/ml). The cells were split 1:1 when they reach $3x10^6$ cells/ml density. After 4–6 days the cells were further stimulated with anti-CD3 and anti-CD28 antibodies (Dynabeads Mouse CD3/CD28 T-cell Expander, Invitrogen) and used for adoptive transfer 10–18 days after the start of the culture.

MethA-EGFP tumor challenge and adoptive cell transfer

F1 Balb/c x C57Bl/6 mice 6 to 12 weeks of age (n = 5–6 for all groups) were injected subcutaneously with 5 x 10^5 MethA-EGFP sarcoma cells and treated with intravenous adoptive transfer of in vitro–activated MethA-EGFP specific T cells derived from parental Balb/c donors as indicated. Lymphopenia was induced by nonmyeloablative cyclophosphamide (CPA) administration (100 mg/kg) one day before the cell transfer. Tumor growth was measured twice weekly in a blinded, randomized fashion. All experiments were performed independently at least twice, with similar results.

Characterization of in vitro expanded T-cells and cell trafficking in vivo

Cellular phenotypes were determined by flow cytometric analysis after staining with antibodies specific for CD3, CD4, CD8, CD49a, CD25, CD69, CD44, CD122, CD127 antigens (BD Pharmingen). For in vivo trafficking T cells were stained in vitro with CellTrace Far Red DDAO-SE (Molecular Probes, Eugene, OR, USA) and injected intravenously to BALB/c mice. Injected mice were analyzed with IVIS Spectrum whole live-animal imaging system (Perkin Elmer Inc., Waltham, MA, USA). Mice were anesthetized with isoflurane using a vaporizer, and fluorescent image was obtained using appropriate filter set.

Histology

Livers, small intestines, and skin from killed ACT recipients were fixed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Images were obtained using a Nikon Eclipse E400 microscope (Tokyo, Japan) equipped with Nuance Multispectral Imaging System VIS and related software (CRI, Woburn, MA). Original magnification was ×100.

Results

To test whether allogeneic tumor-specific T cells could be effectively used to treat tumors, we created an allogeneic adoptive immunotherapy model based on the MethA-EGFP system that we previously described [17]. T cells from MethA-EGFP immune Balb/c mice recognize unidentified MethA tumor antigens as well as the H-2^d-restricted epitope of the EGFP antigen corresponding to amino acids 200 to 208 that is expressed in MethA-EGFP sarcoma tumor [16]. To create a source of tumor-specific allogeneic effector cells, we immunized Balb/c mice (H-2^d) with syngeneic, irradiated MethA-EGFP tumor cells. To assess the ability of allogeneic effector cells to reject tumor cells in MHC-mismatched hosts, we transferred Balb/c derived and in vitro expanded anti-MethA-EGFP T cells into F1 Balb/c x C57BL/6 mice that received 100mg/kg CPA as lymphodepleting regimen before the transfer. The administration of 100mg/kg CPA is a well-established conditioning regimen that induces a profound but transient lymphodepletion lasting for 7 to 10 days [18, 19]. This dose has previously been shown to augment the effectiveness of adoptive cell transfer therapy in various models through several mechanisms, including the removal of immunoregulatory elements, "cytokine sinks", and activation of antigen-presenting cells [18]. Through this approach we wanted to induce a sufficient degree of immunosuppression that would enable the engraftment and in vivo expansion of allogeneic T cells. Since the use of allogeneic T cell has a risk of GVHD, the second objective was to study the influence of allogeneic adoptive cell transfer on GVHD development.

Phenotype of expanded T-cells

T-cell cultures consisted of cells with morphological features typical of activated T cells. The presence or absence of various immune cell types was evaluated by flow cytometric analysis for markers including CD3, CD4, CD8, CD49a, CD25, CD69, CD44, CD122, CD127. As expected, CD3+ T cells were the predominant cell type with majority of cells expressing CD4 marker. CD8+ cells accounted for less than 10% of the cell population. Constantly all cells in culture showed expression of the CD44 antigen which is effector-memory T-cell cell marker. CD25 activation marker was present on 88% of CD8+ and 40% of CD4+ cells. The CD122 antigen 75-kDa subunit of the high-affinity interleukin-2 receptor (IL-2R) was detected on 98% of CD8+ and 80% of CD4+ cells respectively.

Table 1. Immunophenotype of T cells used for ACT

T cell marker	CD3	CD4	CD8	CD25	CD44	CD49a	CD122	CD127
%	99	74	6,5	40 (CD4) 88 (CD8)	99	50	80 (CD4) 98 (CD8)	86 (CD4) 92 (CD8)

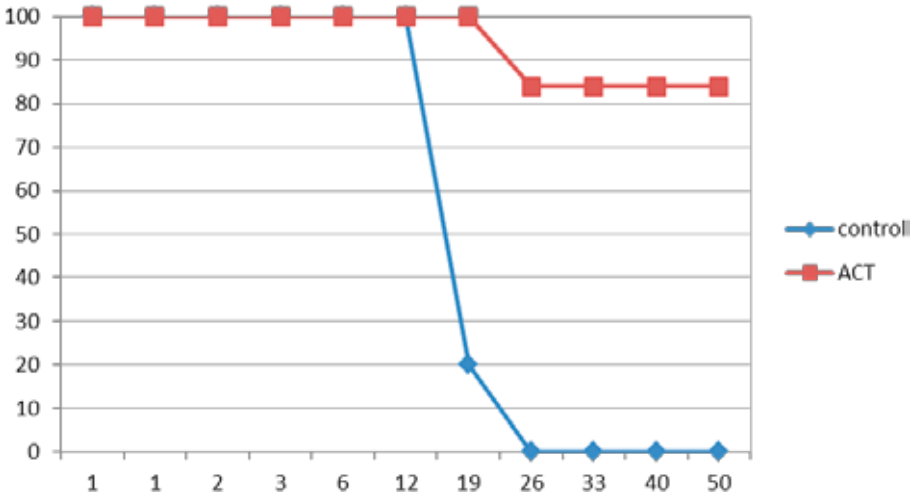

Figure 1. Allogeneic tumor-specific lymphocytes prevent tumor formation. F1 Balb/c x C57BL/6 mice were injected s.c. with MethA-EGFP tumor cells and subjected to ACT with semi-allogeneic in vitro expanded T cell effectors (as indicated). All groups received 100mg/kg of CPA as a preconditioning the day before the transfer of the effector cells (day 1). Results of tumor formation are the observation of at least 5 mice per group. Data are representative of 3 independent experiments

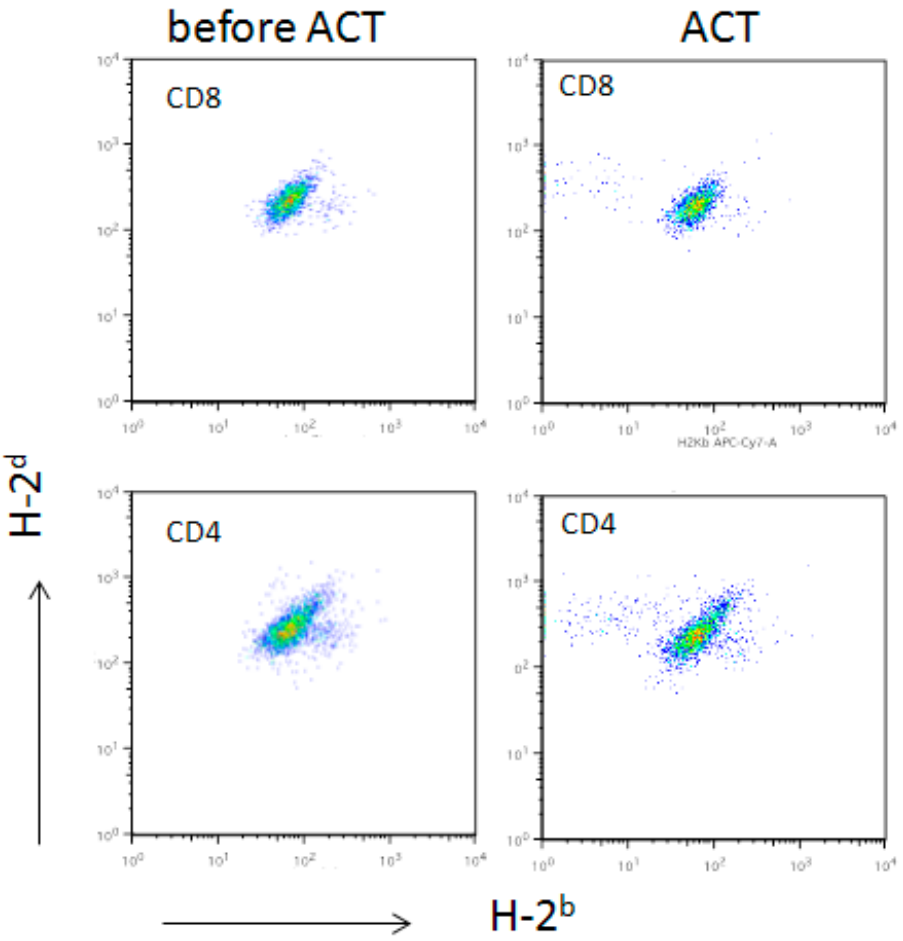

Figure 2. Allogeneic antitumor T lymphocyte persistence in vivo. F1 Balb/c x C57BL/6 mice were treated with $20x10^6$ allogeneic anti-MethA-EGFP T cells. After 21 days, mice were killed, and the spleens were analyzed by flow cytometry for the presence of the transferred cells. The dot plots show the percentage of transferred H-$2^{d/d}$ T cells among predominant H-$2^{b/d}$ host cells. Allogeneic cells were detectable up to day 42 after transfer. Data are representative of 3 independent experiments

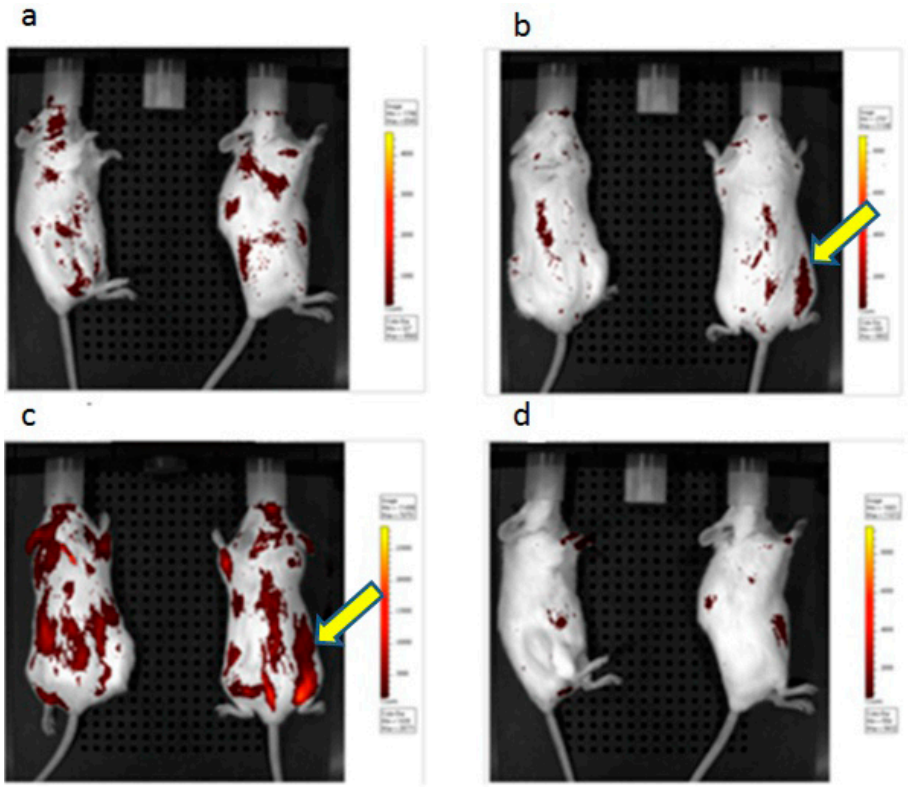

Figure 3. Engraftment, biodistribution and viability of effector cells in vivo. Anti-MethA-EGFP effector cells were expanded in vitro, labeled with CellTrace Far Red DDAO-SE and transferred into MethA-EGFP tumor bearing mice. Images were taken using IVIS-Spectrum Imaging System after 2(a), 24(b), 48(c) and 120(d) hours. Please note selective accumulation at tumor site (arrows, panel b, c)

Allogeneic effector cells prevent tumor formation

The aim of this study was to investigate the ability of allogeneic effector cells to reject implanted tumor cells in vivo. We used Balb/c derived T cells to treat MethA-EGFP-bearing F1 Balb/c x C57BL/6 mice. The exogenous lymphocytes present in the recipient may recognize syngeneic tumor cells and allogeneic MHC molecules expressed by the host organs. This model eliminates the possibility of a rejection of the donor cells because F1 lymphocytes are tolerant to all antigens in the Balb/c background, however GVHD-like reaction from parental donor Balb/c cells against recipients remains efficient. Tumor cells were implanted subcutaneously and recipient mice were preconditioned with 100 mg/kg CPA one day before the transfer.

$5x10^6$ in vitro expanded allogeneic haploidentical effector cells considerably prevented tumor formation without any evidence of GVHD-like reaction. Increasing the dose of allogeneic effector cells by 4-fold (i.e., $2x10^7$ cells instead of $5x10^6$) led to a better tumor treatment still without any measurable toxic effect.

Allogeneic effector cells persist in vivo

In next set of experiments, we analyzed the animals at different time points for the presence of allogeneic T cells expressing the MHC class I H-$2^{d/d}$ molecule. In mice that received CPA pretreatment, allogeneic donor T cells were clearly detectable up to 42 days after transfer. Transferred lymphocytes were also present at the tumor site, indicating that they not only persisted but were capable of trafficking to peripheral sites. These data indicated that allogeneic effector cells can engraft for relatively long periods after a conditioning regimen and preserved the ability to infiltrate tumor tissue.

The risk of inducing a GVHD-like reaction is minimal when the T cell specificity is confined

Alloantigen reactive T cells can lead to severe, life-threating GVHD. Host immune suppression by a lymphodepleting regimen before cell transfer makes this conditions even more plausible [1, 18]. As such, adoptive cell transfer with allogeneic antitumor T cells possess a potential danger for inducing a GVHD-like reaction. Theoretically, because the anti-MethA-EGFP TCRs

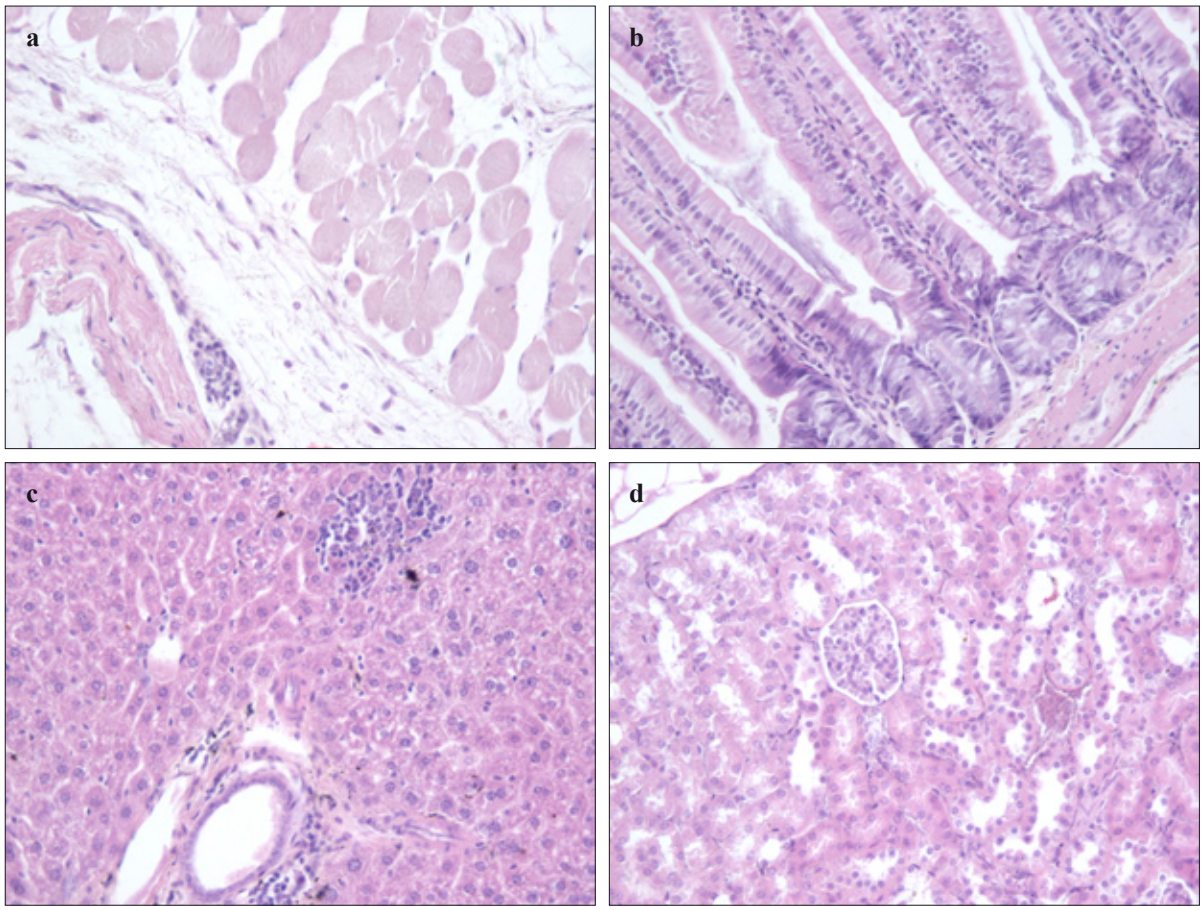

Figure 4. Lack of GVHD-like reactions after parental Balb/c to F1 Balb/c x C57BL/6 tumor specific T cell transfer. In all experiments, in vitro expanded tumor specific T cells were given to cyclophosphamide treated F1 recipients. Skin -A, intestine –B, liver -C, and kidney -D tissue samples were analyzed by histopathology for manifestation of GVHD

are selected on the H-2^d MHC class I molecule, they could cross-react with self-antigens expressed on a different haplotype or even to react with a different MHC class I antigens in a peptide-independent manner [20]. The ability of anti-MethA-EGFP cells to react against recipient cells expressing H-2^b MHC antigens could be tested in vitro with Balb/c cells co-cultured with irradiated splenocytes derived from C57BL/6 or F1 Balb/c x C57BL/6 mice in the presence or the absence of the relevant EGFP peptide. Activation induced CD49b and/or IFN-g expression could be observed only in the presence of the relevant peptide and the proper restriction element (H-2^d), suggesting that the anti-MethA-EGFP TCR cross-reactivity with allogeneic antigens is minimal. However, many more self-antigens are presented in vivo; thus, this kind of in vitro mixed leukocyte reaction might not be an accurate predictor of what would happen after transfer in a living host. A small number of non–tumor-specific T cells might contaminate cell cultures, even after several rounds of in vitro expansions and selection. To address this point, we transferred anti-MethA-EGFP cells into CPA treated semi-allogeneic F1 Balb/c x B6 (H-$2^{b/d}$) or CPA treated syngeneic Balb/c (H-$2^{d/d}$) mice as a control. Transfer of in vitro expanded anti-MethA-EGFP T cells in doses between 5 x 10^6 up to 20 x 10^6 did not result in any measurable toxicity as measured by weight loss, general behavior, survival and histopathology examination. These experiments show that the risk of GVHD is small when allogeneic effector T cells with defined antigen specificity are used.

Discussion

Antitumor T cells has the clinical potential to treat malignancy, both in the setting of autologous TIL for solid tumors and with the use of DLI in hematologic malignancies. Whereas allogeneic adoptive immunotherapy can be quite powerful, many issues remain to be determined for optimization of GVT activity. It is not known whether the GVT reaction is a generalized allogeneic effect or has disease-specific targets, and it

has not yet been possible to consistently separate GVT from GVHD.

In the present study, we tested in animal model the potential use of allogeneic MHC partially matched effector T cells for adoptive tumor immunotherapy. Our results indicate that tumor reactive allogeneic T cells can be obtained and expanded in a relatively easy way, they exhibit antitumor activity in vivo and do not induce severe GVHD. Therefore, this approach seems to be feasible, effective and safe and might bridge allogeneic DLI with autologous TIL therapy.

In our model majority of in vitro expanded allogeneic T cells express CD4+ phenotype, which persists in vivo and mediate antitumor effect despite the absence of MHC class II antigens on tumor cells. Although definitive conclusions are not easy to draw with the currently available data, these findings might be explained by the ability of T helper cells to recruit and activate endogenous effector cells from both the innate and adaptive branch of the immune system to the tumor site [23–25]. In addition, CD4+ T cells through activation of dendritic cells facilitate the priming of specific antitumor CD8+ T cells in the tumor-draining lymph nodes [26–28]. Thus, the requirement for direct tumor recognition might be of lesser importance for antitumor CD4+ T cell–based therapies, making allogeneic T helper cells a particularly viable approach deserving a more detailed investigation.

In our experiments we did not detect any signs of GVHD. Our data indicate that the risk of adverse reaction because of "off target" recognition may be low if antigen specific T cells are used. The risk of severe life threating GVHD increases with the number of T cells infused. Patients treated with high-dose donor lymphocyte infusion developed severe adverse reactions and died of GVHD and bone marrow failure [36]. Thus, it might be useful either to screen allogeneic T-cell effectors and remove those cells that are alloreactive or alternatively expand tumor specific T cells [36–38]. In our study antitumor T cells generated from immune mice had effector memory phenotype and due to define antigen specificity possess a limited TCR repertoire, therefore the risk of GVHD in this setting should be considerably lower even with high cell number. Several reports have shown that naive T cells are much more efficient in initiating graft-versus-host reaction compared with memory T cells [40–43].

In conclusion: there are several reasons that make antigen specific allogeneic adoptive T cell transfer appealing. Relatively easy and unsophisticated method of in vitro preparation ensures improved selection, standardization, quality and safety controls required for clinical products. For selected and common haplotypes the establishment of a bank of highly reactive tumor-specific T cells would allow for massive or standardized treatment. Moreover, multiple, repetitive administrations might be also possible.

Taken together, our findings show that allogeneic T cells might have a potential role in the cell-based immunotherapy of cancer.

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Acid-base balance in acute ethylene glycol poisoning in rats treated with fomepizole

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ABSTRACT

Introduction. Ethylene glycol (EG) is relatively nontoxic but undergoes a multi-step oxidation to toxic metabolites, aldehydes and acids. The accumulation of organic acids, mainly glycolates, leads to the development of profound, life-threatening metabolic acidosis. A key therapy is an antidotal treatment with fomepizole (4-MP), the inhibitor of the first step of EG biotransformation enzyme, alcohol dehydrogenase.

Aim. The aim of the study was to demonstrate the efficacy of fomepizole in the prevention of acid-base balance disorders in acute ethylene glycol poisonings in rats.

Material and methods. Adult male Wistar rats were given EG (*p.o.*) with single (*i.p.*) or multiple (*p.o.*) doses of 4-MP (EG 3830 and 5745 mg/kg, respectively, 4-MP in single dose of 10 mg/kg or 15 mg/kg followed by 10 mg/kg every 12 hours). Blood gas analysis was performed and blood pH, bicarbonate concentration and base excess were evaluated.

Results and conclusions. The single dose of 4-MP was effective in preventing a decrease in blood pH, bicarbonate concentration and base excess during the entire experimental period (pH 7.35 vs 7.21 at hour 12, bicarbonate concentration 27.2 vs 18.3 mmol/dm^3 at hour 8, base excess 1.8 vs -8.2 mmol/dm^3 at hour 18). The multiple administration of 4-MP started 2 hours after EG poisoning resulted in rapid restoration of proper values of acid--base balance parameters. Fomepizole is highly efficacious in restraining the acid-base balance disorders which are concomitant with acute ethylene glycol poisonings.

Key words: ethylene glycol, fomepizole, acid-base balance, acute poisoning, rats.

Introduction

Acute ethylene glycol (EG) poisoning is most frequently a result of ingestion of its solutions (antifreeze, coolants and de-icing fluids), which may be accidental or, in humans, intentional – suicidal or ingested as a substitute for ethanol [1, 2]. Upon absorption to blood EG is relatively nontoxic and in the first stage of poisoning it causes inebriating effects similar to those of ethanol. EG is converted by alcohol dehydrogenase (ADH) and subsequently by other enzymes to highly toxic metabolites, aldehydes and acids [2, 3, 4]. The accumulation of organic acids, mainly glycolates, leads to the development of metabolic acidosis [1, 2, 5]. The reactions of EG oxidation are accompanied by an increase in the ratio of reduced to oxidized form of the nicotinamide adenine dinucleotide, and, consequently, by lactate accumulation and development of lactic acidosis [1, 2].

The development of acid-base balance disorders is typical of the second stage of EG poisoning, which causes multi-organ injury, cardiac dysrhythmia, myocardial depression, congestive heart failure, pulmonary oedema and adult respiratory distress syndrome. Untreated acid-base balance disorders are often fatal [1, 2]. The third stage of poisoning is characterized by acute renal failure due to the accumulation of EG metabolite crystals, calcium oxalate, and the direct cytotoxic effect of other EG metabolites [2, 4].

A first-line therapy in EG acute poisoning is an antidotal treatment with ADH inhibitor, ethanol or fomepizole, which enables excretion of the unchanged EG

with urine [2, 3]. Fomepizole (4-methylpyrazole, 4-MP) is a newer, more efficacious and safer antidote in poisonings caused by EG, methanol and other toxic alcohols [2, 3, 6, 7].

A review of available literature provides only limited information about the influence of the treatment with fomepizole on the acid-base status in acute ethylene glycol poisoning. These data come from studies conducted on small groups of animals, such as dogs or cats [8, 9]. The aim our study was to demonstrate the efficacy of 4-MP in the prevention of acid-base balance disorders in acute ethylene glycol poisonings in rats.

Material and methods

Material

Ethylene glycol 99%+ (Sigma-Aldrich) and 4-methylpyrazole 99% (Aldrich) were used in the experiment. Solutions were prepared with use of *Aqua pro injectione* and *Natrium chloratum 0.9% inj.* (Baxter Polska).

Animals

Adult male Wistar rats weighing 280 ± 20 g were purchased from the breeding farm of the Department of Toxicology, Poznan University of Medical Sciences, Poznań, Poland. The animals were fed with standard laboratory chow Labofeed H and were provided drinking water *ad libitum*. The room in which the animals were housed had standardized environmental temperature (20 ± 2°C), relative humidity (50–60%) and 12-h photocycle. Food was withdrawn 12 hours prior the administration of xenobiotics and rats were starved during the whole experimental period in stage I. In stage II the food was returned 6 hours post-dosing. Water *ad libitum* was provided all the time.

Animals used in the study were treated in compliance with the recommendations from the Declaration of Helsinki. The experimental protocol was approved by the Local Ethical Committee for Experiments on Animals (permissions no. 3/2004 and 20/2009).

Experimental design

There were two separate stages of the study.

Stage I – the animals were divided into 4 groups: control group K1 receiving water, group G1 receiving ethylene glycol at a dose of 3830 mg/kg (*p.o.*), group M1 receiving fomepizole at a dose of 10 mg/kg (*i.p.*) and group GM1 receiving ethylene glycol at a dose of 3830 mg/kg (*p.o.*) together with fomepizole at a dose of 10 mg/kg (*i.p.*). Each of the groups consisted of 55 animals, 5 for each of 11 time points of the experiment.

The xenobiotics under study were administered once and simultaneously. The animals in control group K1 were given 3 cm^3 of water by gavage and the animals in groups G1 and GM1 were given ethylene glycol by gavage as a 3 cm^3 water solution. The animals in groups M1 and GM1 were given fomepizole intraperitoneally as a 0.5 cm^3 solution in saline.

Stage II – the animals were divided into 3 groups: control group K2 receiving water, group G2 receiving ethylene glycol at a dose of 5745 mg/kg (*p.o.*) and group GM2 receiving ethylene glycol at a dose of 5745 mg/kg (*p.o.*) together with fomepizole at a loading dose of 15 mg/kg (*p.o.*) 2 hours after EG administration, followed by maintaining doses of 10 mg/kg (*p.o.*) every 12 hours. Each of the groups consisted of 105 animals, 7 for each of 15 time points of the experiment.

The animals in control group K2 were given 3 cm^3 of water by gavage and the animals in groups G2 and GM2 were given ethylene glycol by gavage as a 3 cm^3 water solution. The animals in the group GM2 were given fomepizole as a 1 cm^3 water solution.

In both stages the animals were anesthetized with ketamine (Bioketan®, Biowet, Poland, 100 mg/kg, *i.m.*) after 0.5, 1, 2, 4, 6, 8, 12, 18, 24, 36 and 48 hours and additionally after 60, 72, 96 and 120 hours in stage II. Blood was collected from the rat tail into heparinised capillary tubes.

Blood gas analysis

Blood gas analysis was conducted on Radiometer ABL 500 blood gas analyzer. The device was equipped with electrodes for pH, oxygen tension and carbon dioxide tension measurements. On the basis of the results of determined parameters the analyzer calculated the bicarbonate concentration and base excess (BE).

Statistical analysis

The received results are presented as an arithmetic mean. All parameters were compared between the groups for each of the time points by one-way ANOVA with Tukey's *post hoc* test. The statistical analysis was made with GraphPad InStat ver. 3.06 for Windows (GraphPad Software). Values of $P < 0.05$ were considered significant. Data different from the values in the control group are marked with an asterisk (*) and data different from the values in the untreated groups poisoned with ethylene glycol are marked with a dagger (†).

Results

Figures 1–3 show the results of blood gas analysis in stage I concerning blood pH, bicarbonate concentration and BE after EG administration to the rats (3830 mg/kg, *p.o.*) and/or 4-MP (10 mg/kg, *i.p.*).

In control group K1 in 0.5–48 hours blood pH ranged from 7.35 to 7.40, bicarbonate concentration 29.6–33.3 mmol/dm^3 and base excess 4.7–8.1 mmol/dm^3. In group G1 blood pH decreased significantly after EG administration as early as 1 hour of the experiment (pH 7.26), reaching the minimum after 12 hours (pH 7.21). After 48 hours blood pH returned to the reference values (pH 7.36). Similarly, the bicarbonate concentration was decreased, reaching the minimum (18.3 mmol/dm^3) after 8 hours of the experiment. Normal values were found again after 36 and 48 hours post-dosing (24.9 and 32.3 mmol/dm^3, respectively). BE reflected measured bicarbonate concentrations and reached -7.9 mmol/dm^3 after 6 hours and a minimum of -8.2 mmol/dm^3 18 hours after EG administration. At the end of the experiment BE ranged between the reference values (-1.2 mmol/dm^3 after 36 hours and 6.9 mmol/dm^3 after 48 hours).

In groups M1 and GM1 blood pH was slightly lower than in the control group, with a significant decrease after 2 and 6–18 hours in group GM1. At all time points the blood pH values did not fall below 7.31. The bicarbonate concentration (minimum 26.0 mmol/dm^3) and BE (minimum 0.6 mmol/dm^3) behaved similarly in these groups.

Figures 4–6 show the results of blood gas analysis in stage II concerning blood pH, bicarbonate concentration and BE after EG administration to the rats (5745 mg/kg, *p.o.*), single and combined with 4-MP at the loading dose of 15 mg/kg (*p.o.*) 2 hours after EG administration followed by maintaining doses of 10 mg/kg (*p.o.*) every 12 hours.

In control group K2 in 0.5–120 hours blood pH was ranged from 7.34 to 7.38, bicarbonate concentration 27.1–32.9 mmol/dm^3 and BE 3.5–6.5 mmol/dm^3. In group G2 blood pH after the administration of ethylene glycol was significantly lower, starting from 0.5 hour (pH 7.28) to 36 hours, reaching the minimum 4 hours post-dosing (pH 7.20).

After 48 hours of the experiment blood pH almost returned to reference values (pH 7.33). The bicarbonate concentration was decreased analogically, reaching the minimum (19.3 mmol/dm^3) after 4 hours. Normal values were recovered after 24 hours of poisoning (31.2 mmol/dm^3). The measured base excess was minimal (-8.9 mmol/dm^3) 4 hours post-dosing and after 24 hours it returned to the reference values (3.7 mmol/dm^3).

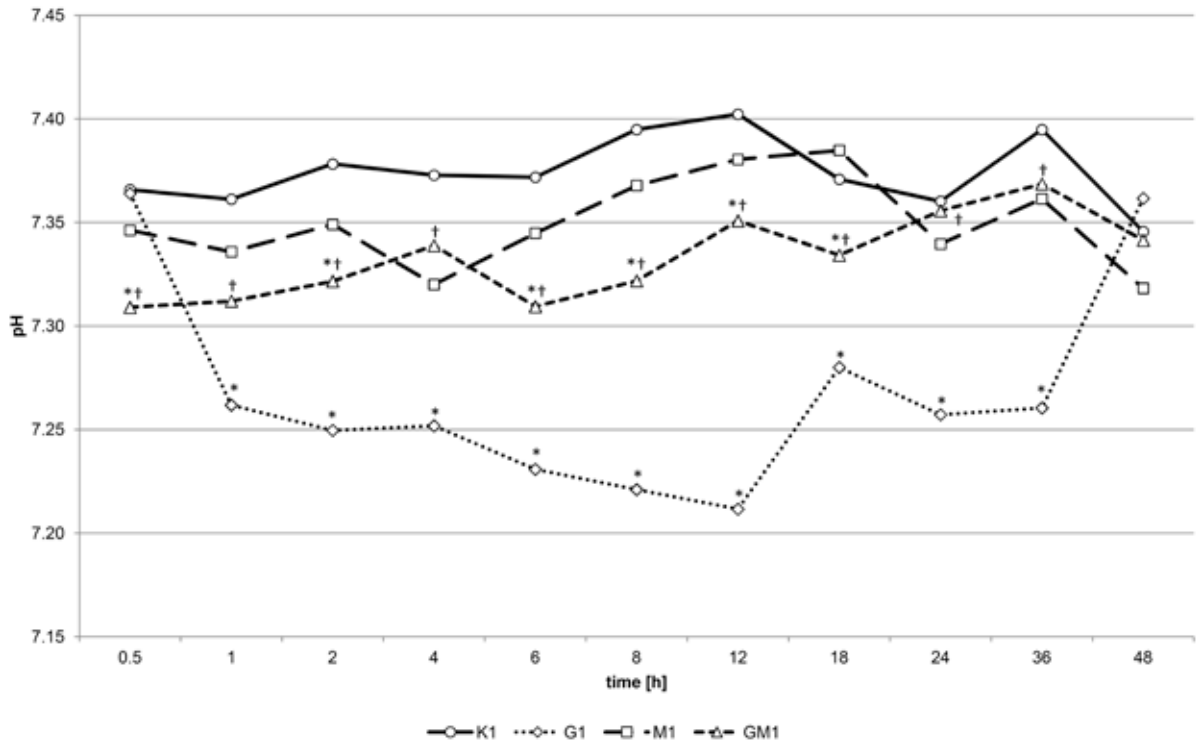

Figure 1. Blood pH in rats – stage I

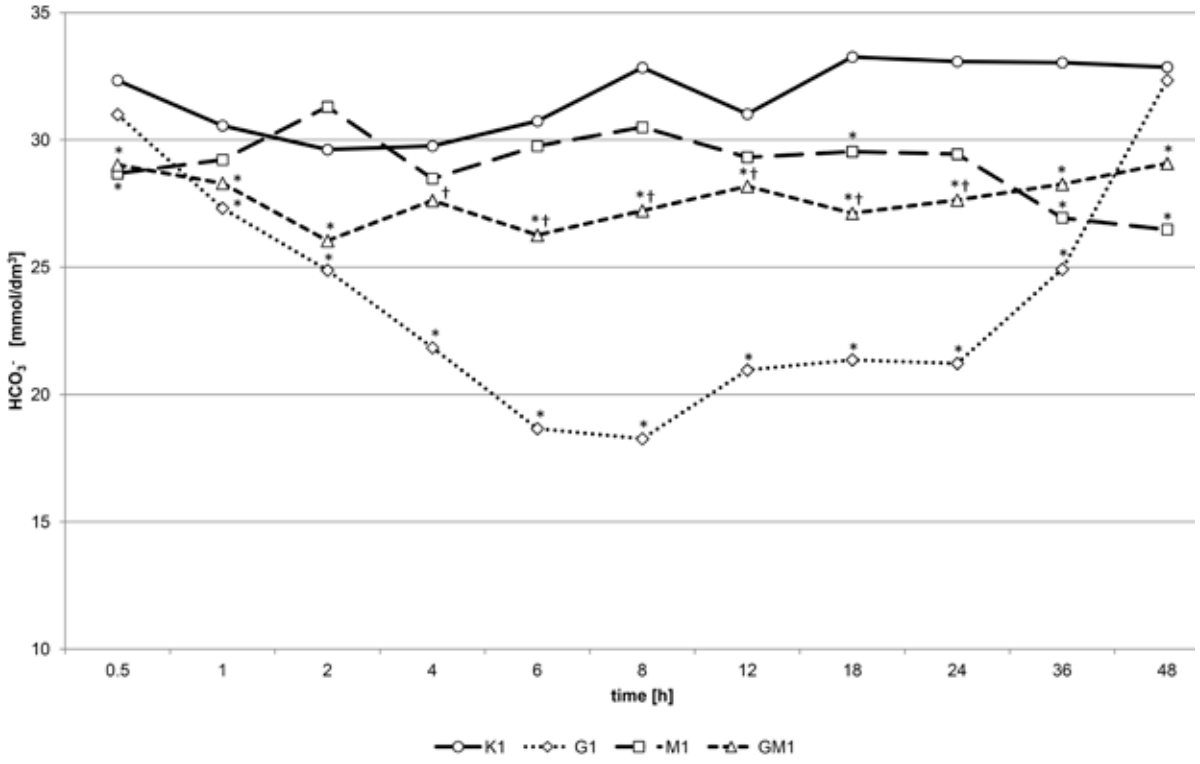

Figure 2. Blood bicarbonate concentration in rats – stage I

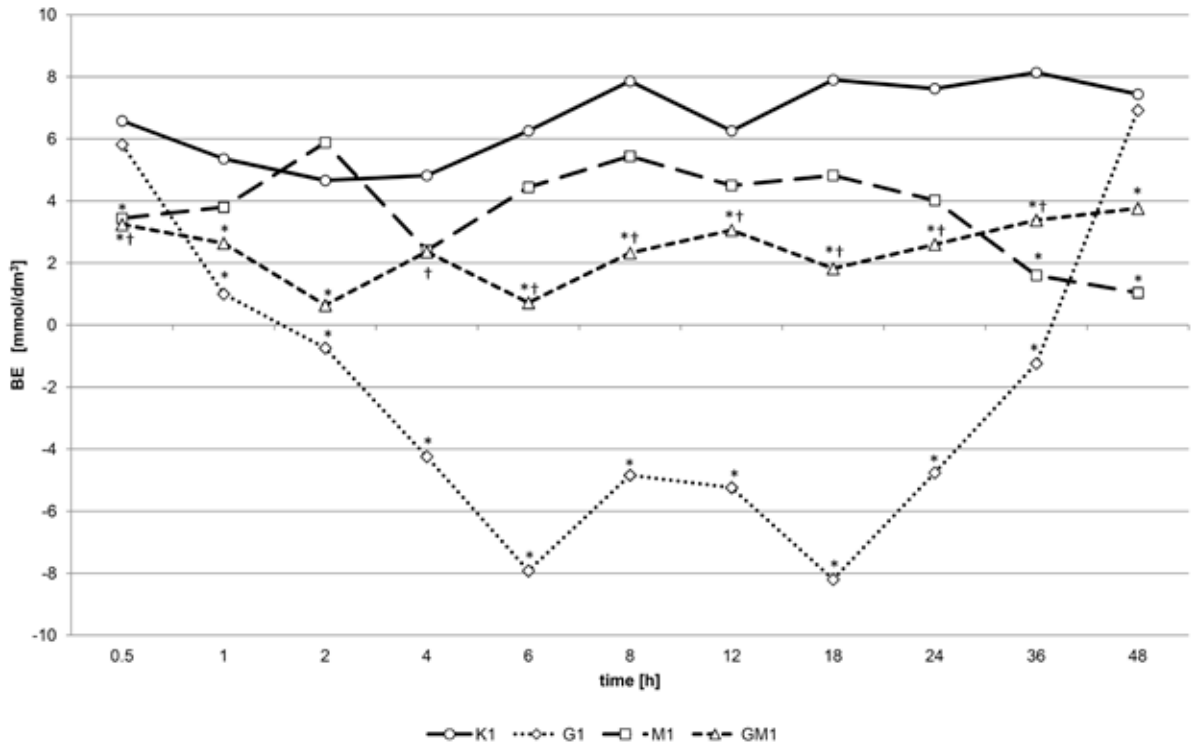

Figure 3. Blood base excess in rats – stage I

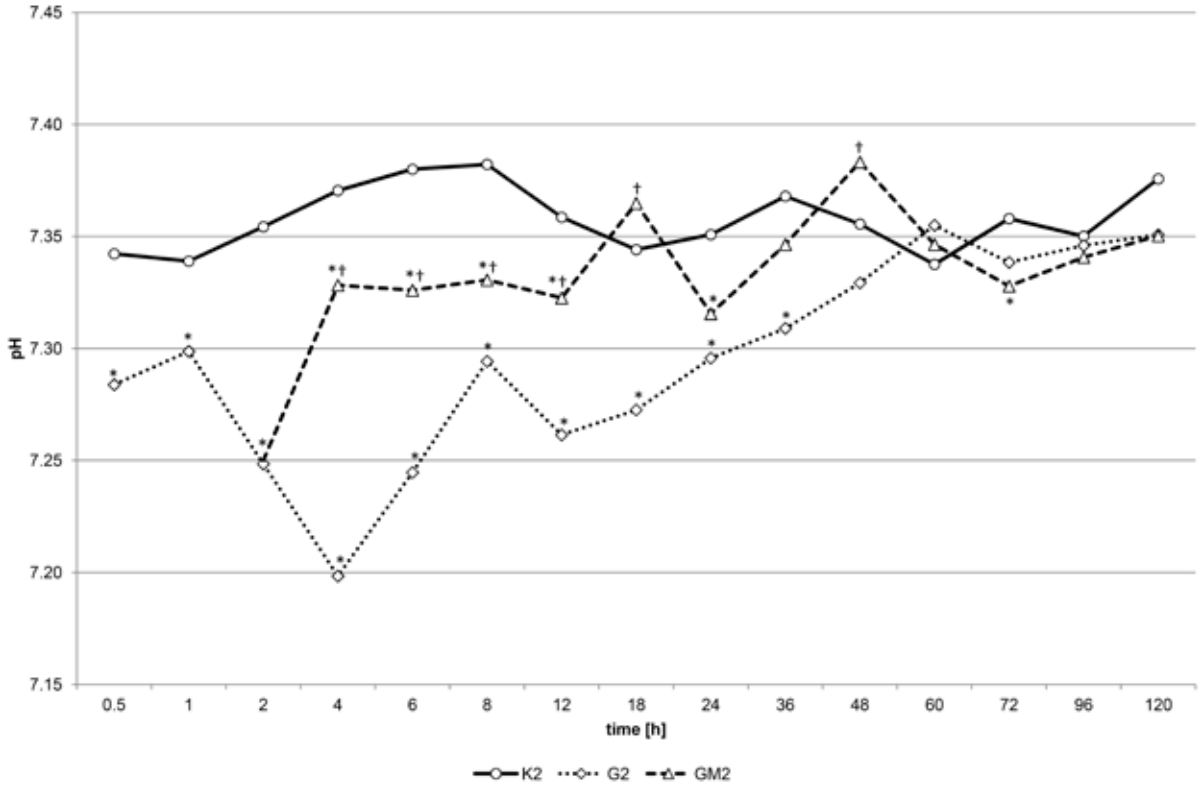

Figure 4. Blood pH in rats – stage II

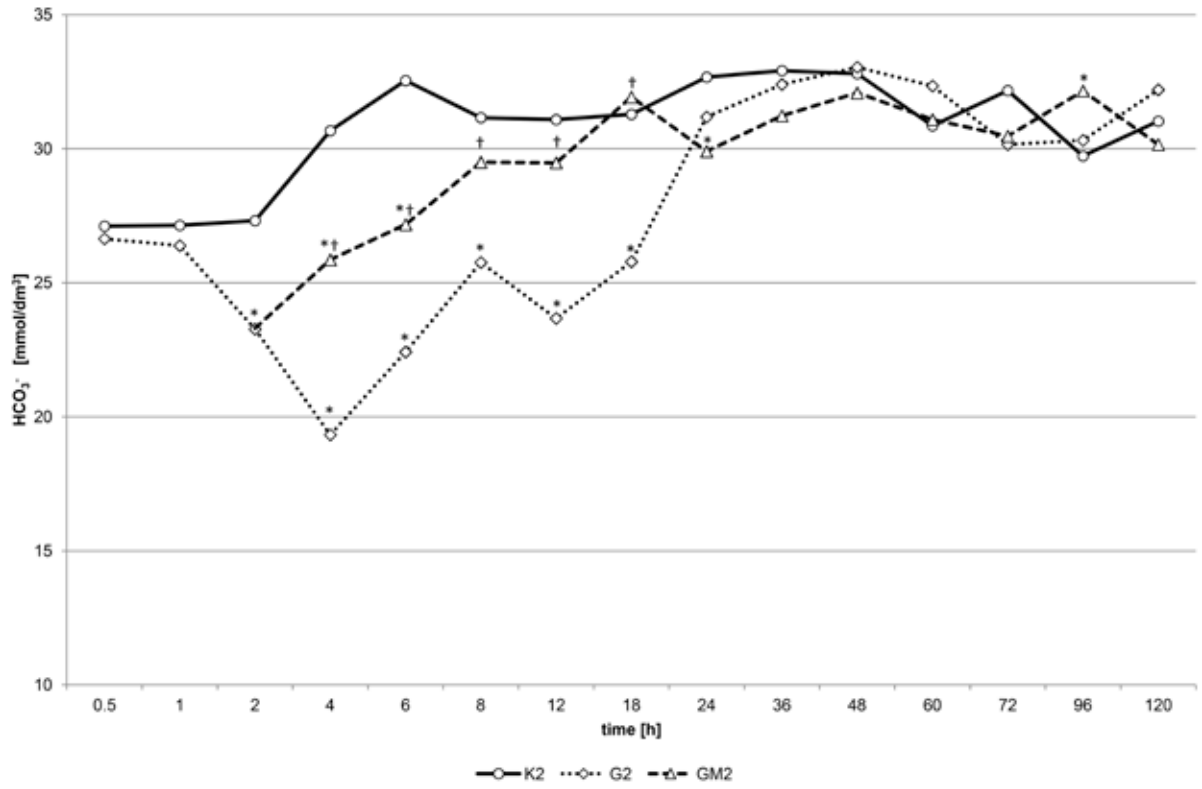

Figure 5. Blood bicarbonate concentration in rats – stage II

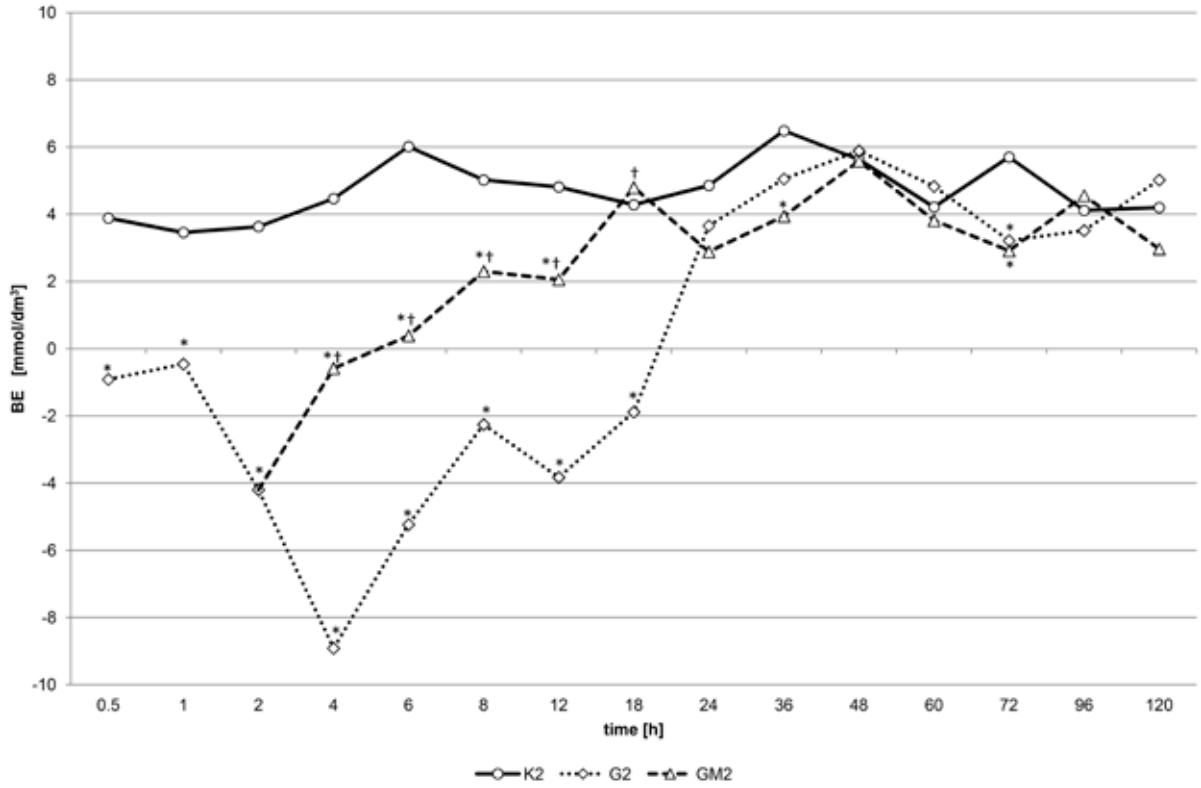

Figure 6. Blood base excess in rats – stage II

The administration of 4-MP at a loading dose 2 hours after EG poisoning in group GM2 resulted in a statistically significant increase in all of the evaluated parameters (pH 7.33 vs 7.20, HCO_3^- 25.9 vs 19.3 mmol/dm^3, BE -0.6 vs -8.9 mmol/dm^3) as early as 2 hours later, i.e., 4 hours after the administration of EG, as compared to untreated group G2. After 18 hours of the experiment the values of acid-base balance parameters in group GM2 did not differ significantly from the values in control group K2, except the time point of 24 hours, at which they temporarily decreased slightly (pH 7.32, HCO_3^- 29.9 mmol/dm^3, BE 2.9 mmol/dm^3).

Discussion

The development of metabolic acidosis is typical of ethylene glycol poisoning and is associated with EG biotransformation to acidic metabolites, above all with the production and accumulation of glycolates, and to a lesser extent – glyoxylates and oxalates [2, 10]. During the multi-step reaction of EG oxidation the nicotinamide adenine dinucleotide (NAD) is depleted, which results in the increase in $NADH+H^+/NAD^+$ ratio and, subsequently, in the increase in lactate/pyruvate ratio, contributing to the development of lactic acidosis [11]. The bicarbonate buffering system, the main buffering system of the extracellular fluid, is the first line of defence against the arising acidic EG metabolites and pH disorders developing under their influence [12, 13]. The biotransformation of glycolates to glycine, lactate and carbon dioxide is also responsible for the decrease in bicarbonate concentration [10, 14]. The development of acidosis is facilitated by the disturbed respiratory compensation of metabolic acidosis due to respiratory depression and by the progression of renal failure [10, 12].

In humans, EG acute poisoning with a decrease in bicarbonate concentration below 9 mmol/dm^3 considerably increases the risk of death and a drop below 5 mmol/dm^3 causes the risk of death to be 3 times more likely than survival. The risk of death is similarly increased when pH falls below 7.0 [4]. The drop of blood pH below 6.8 was demonstrated to result in protein denaturation, inhibition of enzymes and it may lead to death [15]. The lowest values of pH and bicarbonate concentrations mentioned in the literature in patients who had survived acute EG poisoning were 6.46 [14] and 0.5 mmol/dm^3 [16], respectively.

To assess the acid-base balance in untreated and treated acute ethylene glycol poisoning in rats pH,

bicarbonate concentration and base excess in capillary blood were measured.

Blood pH in control groups K1 and K2 ranged from 7.34 to 7.40 during the whole experiment and almost coincided with the reference values in humans which vary from 7.36 to 7.44 [17]. The bicarbonate concentration in these groups was 27.1–33.3 mmol/dm^3, so it was higher than so-called standard bicarbonate concentration – 21–25 mmol/dm^3 [17]. Base excess in animals from control groups (3.5–8.1 mmol/dm^3) was also elevated in comparison with the reference values in humans 0 ± 2.3 mmol/dm^3 [17].

EG administration in stage I and stage II caused a rapid and statistically significant decrease in blood pH, bicarbonate concentration and base excess since the first hours of the experiment. These changes signify the occurrence of metabolic acidosis. All the animals survived and the values of parameters under analysis began to rise to reach the reference values of the control groups at the end of the second day of the experiment. Olszowy [10] described similar results in Wistar rats in which the maximum decrease in blood pH and bicarbonate concentration occurred as early as 2 hours and returned in 10 hours after oral administration of EG at a dose of 3830 mg/kg. Hewlett et al. [18] demonstrated the lowest bicarbonate concentration 6 hours post-dosing in Sprague-Dawley rats (EG 2000 mg/kg, *p.o.*). Subcutaneous EG administration at a dose of 3333 mg/kg to female Sprague-Dawley rats resulted in a decrease in blood pH to 7.12 9 hours after administration [19]. More profound metabolic acidosis was described in pigtail monkeys *Macaca nemestrina*, which received EG at a dose of 4000 mg/kg intraperitoneally. The lowest values of blood pH and bicarbonate concentrations were determined 12–15 hours post-dosing, 7.13–7.18 and 5.5–8.2 mmol/dm^3, respectively [5]. A similar bicarbonate concentration after 12 hours (8.1 mmol/dm^3) was found in dogs after oral EG administration at a dose of 10740 mg/kg [9].

The time of occurrence of the most profound metabolic acidosis may be connected with the moment of peak concentration of acidic EG metabolites, mainly glycolates [1]. The authors' own studies of pharmacokinetics of EG and its metabolite revealed the maximum glycolate concentration 4 hours (stage I, 0.61 g/dm^3) and 4–6 hours (stage II, 0.68–0.75 g/dm^3) after EG administration (unpublished data). Similar maximum glycolate concentration and time of its occurrence were described by Hewlett et al. [18] after oral administration of ethylene glycol to Sprague-Dawley rats at a dose of 2000 mg/kg.

The main goal of the treatment with 4-MP in acute EG poisoning is the inhibition of ADH, resulting in an increase in urinary EG excretion due to the inhibition of this metabolic route. Therefore, fomepizole administration should prevent the development of metabolic acidosis and multi-organ injury.

In the animals which were given single 4-MP and in combination with EG in stage I as well as they were given 4-MP 2 hours after EG administration and every consecutive 12 hours in stage II, the values of blood pH, bicarbonate concentration and BE were slightly lower than the reference values in the control groups. In groups GM1 and GM2 all the acid-base balance parameters under analysis at most time points were significantly higher than the values in the untreated animals, i.e., in groups G1 and G2, which received EG only. The administration of 4-MP 2 hours after EG poisoning in group GM2 caused a statistically significant increase in blood pH, bicarbonate concentration and BE as soon as 4 hours after poisoning, as compared with the untreated group G2 (pH 7.33 vs 7.20, HCO_3^- 25.9 vs 19.3 mmol/dm^3, BE -0.6 vs -8.9 mmol/dm^3). The obtained results after simultaneous administration of EG and 4-MP (stage I) as well as after a 2-hour delay in 4-MP administration (stage II) proved 4-MP to be efficacious and rapid in preventing the development of metabolic acidosis. The therapeutic effect in stage II was achieved already after the administration of the first dose of 4-MP 2 hours after poisoning and the next maintaining doses given every 12 hours enabled the monitored parameters to recover the reference values after 24 hours of the experiment.

Similar results were reported in a study on dogs (EG 10740 mg/kg, *p.o.*). Fomepizole administration in one dose of 20 mg/kg 3 hours after EG poisoning caused an increase in bicarbonate concentration after 6 hours to 21.9 mmol/dm^3 vs 12.6 mmol/dm^3 in the untreated group [9]. Connally et al. [8] demonstrated the return of blood pH and bicarbonate concentration to reference values after 24 and 48 hours, respectively, in cats poisoned with a lethal dose of ethylene glycol and treated with 4-MP 3 hours after EG poisoning (initial dose of 125 mg/kg and maintaining dose of 31.3 mg/kg every 12 hours). In humans poisoned with EG, who were treated with 4-MP, blood pH was ranged from 7.14 to 7.34 and bicarbonate concentration was ranged from 6.8 to 17.8 mmol/dm^3. Lower values were noted in patients with renal insufficiency [20].

In conclusion, the administration of a single dose of fomepizole at a dose of 10 mg/kg with a simultaneous administration of ethylene glycol at a dose of

3830 mg/kg was effective in preventing a decrease in blood pH, bicarbonate concentration and base excess during the entire experimental period.

The administration of 4-MP 2 hours after EG poisoning (5745 mg/kg) at a loading dose of 15 mg/kg and every 12 hours at maintaining doses of 10 mg/kg resulted in rapid restoration of proper values of acid-base balance parameters.

The obtained results let us conclude that fomepizole is highly efficacious in restraining the acid-base balance disorders which are concomitant with acute ethylene glycol poisonings.

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Immunocytochemical indicators of apoptosis in gingival tissues of patients with chronic periodontitis

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ABSTRACT

Introduction. Inflammatory mechanisms of chronic periodontitis (CP) may be linked to various forms of disturbances in apoptosis.
Aim. The study aimed at comparison of tissue expression of anti-apoptotic protein (Bcl-2) and proapoptotic proteins (p53, caspase-3) in gingival tissues of 30 patients with CP and of 15 with healthy periodontium.
Material and methods. Gingival samples (n = 68) were obtained during the open curettage procedure with gingivectomy of adult patients (18 women and 12 men) with CP. Classical immunocytochemical (IHC) method was used to detect apoptotic proteins, and the obtained expression was evaluated using semi-quantitative IRS scale.
Results. No differences could be revealed in expression intensity or reciprocal correlations between apoptotic proteins within the group of patients with CP. Greater expression of the two apoptotic proteins (Bcl-2 and p53) were detected in patients with CP than in control individuals. Moreover, a more pronounced expression of Bcl-2 was demonstrated in gingival samples of patients with localised form as compared to generalised form of CP. Expression of caspase-3 (effector phase of apoptosis) manifested no differences between CP and control individuals. Greater expression of the anti-apoptotic protein Bcl-2 and caspase-3 was detected in cells of inflammatory infiltrates in lamina propria than in keratinocytes.
Conclusions. In CP significant alterations developed in expression of indicators of apoptosis, with prevalence of Bcl-2 and p53 expression, as compared to the control. The localised form of CP was linked to higher proportion of Bcl-2-positive cells of inflammatory infiltrates, suggesting that apoptosis was inhibited mainly in this form of CP. The comparable expression of caspase-3 in gingival cells with CP and in control and absence of correlation with clinical data suggested that the process of apoptosis did not play a significant role in destruction of periodontium tissues in CP.

Key words: chronic periodontitis, apoptosis, immunocytochemistry.

Introduction

Chronic periodontitis (CP) develops due to bacterial and/or viral infection and is accompanied by a progressive loss of clinical attachment level/loss (CAL) and of alveolar bone. The process represents one of principal causes of dental loss in adult patients [1–3]. It is estimated that in various world populations 1–5% individuals suffer from aggressive periodontitis and 80% from chronic periodontitis, which allows to consider CP as representing a social problem [4]. Several hypotheses exist related to pathomechanisms of the disease [3–5]. Involvement of bacteria which are pathogenic for periodontal tissues (*A. actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannarella forsythensis)* in promotion of cell apoptosis was confirmed by numerous studies in the *in*

vitro models and in the *in vivo* conditions [6–12]. The most typical for apoptosis morphological lesions in cell nuclei (increased fragmentation of DNA, the typical DNA „ladder") were induced by *Tannarella forsythensis* (earlier termed *B. forsythus*) and serotype „a" of *A. Actinomycetemcomitans* [6]. *Porphyromonas gingivalis* induced a direct cytopathic effect [13, 14]. Apoptosis induced by infection with the bacteria was demonstrated in several cell types, *i.e.* in peripheral blood mononuclear cells (PBMCs) [13], lymphocytes [14], epithelial cells [9, 11, 12], fibroblasts and endothelial cells of gingival blood vessels [15–18] and in macrophages [6, 12]. The *in vitro* models explained role of bacterial factors engaged in the apoptotic process in periodontitis. A proportion of authors indicated that gingipains (Arg-gingipain, and Lys-gingipain) and endopeptidase O (PepO) may induce alterations in adhesion proteins and may promote apoptosis of fibroblasts and vascular endothelial cells [15–17]. A team of polish investigators demonstrated that gingipains influenced the immune system inducing degradation of TNF-α, which resulted in, *i.a.*, dysregulation of cytokine network and inhibition of activity manifested by transcription factor, NF-κB [19]. Other investigators showed that apoptosis in primary human gingival fibroblasts (HGF) in infection with *P. gingivalis* developed in two stages and involved a variable in time regulation of anti- and proapoptotic molecules [18]. The authors confirmed the earlier observations that gingipains may directly promote apoptosis through the intracellular proteolytic activation of caspases.

Other studies related to the role of apoptosis in periodontitis posed the question related to the role played by inhibition of apoptotic process in chronic transformation of pathology in CP. According to certain authors, the inhibition of apoptosis in, *e.g.*, macrophages may reflect a decreased production of interleukin 4 (IL-4) by cells of inflammatory infiltrates [20]. Other authors described the phenomenon of resistance to apoptosis manifested by infiltrating lymphocytes (absence of an appropriate expression of Fas ligand at the cell surface with preserved function of Fas receptor) [21]. Subsequent studies demonstrated induction of the anti-apoptotic Bcl-2 protein in gingival keratinocytes under effect of *P. gingivalis* [22]. Yilmaz et al. provided the observation that *P. gingivalis* at first triggered surface phosphatidylserine exposure through a mechanism requiring caspase activation, but after 1 day of infection protected infected cells against apoptosis [23].

Using immunocytochemical (IHC) techniques apoptotic proteins in CP were demonstrated both in epithelial keratinocytes and in cells of inflammatory infiltrates in *lamina propria* [8–10, 14, 24]. A more pronounced expression of proapoptotic proteins was manifested in gingival samples of CP patients than in a control group [8, 14]. Studies related to the active phase of apoptosis suggested that activation of caspases (-3, -7 and PARP) represents the principal trait of tissue injury in the course of periodontitis and it affects both keratinocytes and connective tissue cells [10].

This study aimed at comparison of expression manifested by key apoptotic proteins, *i.e.*, the anti-apoptotic Bcl-2 protein and the two pro-apoptotic proteins, p53 (early phase of apoptosis) and caspase-3 (effector phase of apoptosis) in gingival samples obtained from patients with CP and from persons with clinically healthy periodontium, as related to selected clinical data.

Material and methods

Clinical data of CP patients and of control individuals are given in Tables 1 and 2. The studies were conducted on the total of 17 patients with generalised form of CP and 13 patients with localised CP. The parameter defining clinical advancement of the disease involved clinical attachment level/loss (CAL), estimated using Florida Probe®. The system consists of a periodontological optical probe PD/CEJ and a computer software FP32 v.6. The PD/CEJ (10.8x0.45 mm STANDARD probe) manifests the accuracy of 0.2 mm at the pressure corresponding to weight of 15 g. The values of CAL I, II and III were demonstrated in, respectively, 7, 19 and 42 fragments of gingivae obtained from CP patients (Table 1).

Tissue material

Gingival samples (n = 68) from 30 adult patients (18 women and 12 men; mean age: 45 ± 2 years) were isolated using a scalpel from various sextants, from proximity of various maxillary and mandibular teeth (mainly incisors, canines and premolars) during the open curretage with gingivectomy, following a successful completion of hygienic phase of the periodontal therapy (API index <15%) (Table 1). In every patient 1–4 fragments of periodontal tissues were sampled from proximity of various teeth with various values of CAL: CAL I $\leqslant$ 3 mm, CAL II 4–6 mm, CAL III $\geqslant$7 mm. The control material (negative control) included gingival samples isolated from 15 patients with clinically healthy periodontium, the gingival fragments of which were sampled during procedures of esthetic periosur-

Table 1. Selected clinical data on patients with chronic periodontitis (CP) and semiquantitative evaluation of tissue expression of apoptotic proteins (Bcl-2, p53, caspase-3) in gingival samples

Patient	Age (years); sex	CP form	Proximity of tooth no.	CAL	Grading	Bcl-2*	p53*	caspase-3*
1.	47; M	G	21	III	3	0/6	nt	0/3
			11	III	3	0/6	nt	0/3
			12	III	3	0/9	6/3	0/6
2.	52; F	L	33	I	2	nt	nt	nt
			31	III	1	0/6	4/3	3/3
			41	III	1	nt	6/6	6/9
3.	47; M	G	22	II	3	0/3	nt	0/9
			11	III	3	0/3	0/2	0/6
			14	III	2	0/4	nt	0/3
			33	III	3	0/3	6/2	0/3
			41	III	3	3/4	6/0	0/9
			43	III	3	0/12	0/3	2/4
4.	49; F	G	42	II	1	0/0	nt	nt
			32	III	3	0/6	nt	0/3
			31	III	3	0/8	3/3	0/3
			41	III	2	nt	0/0	0/2
5.	20; F	L	22	I	2	6/8	0/3	0/6
			11	II	2	0/2	nt	0/0
			13	II	2	3/3	3/3	0/0
6.	49; M	L	31	III	3	2/6	6/3	2/2
			33	I	3	0/4	6/4	0/3
			41	III	3	0/6	6/0	0/9
7.	63; F	G	44	I	2	4/0	0/3	0/9
			43	III	3	0/9	0/3	0/3
			42	III	2	0/0	3/0	6/6
			33	III	2	0/0	0/3	0/8
8.	28; F	L	37	II	1	0/8	0/0	0/3
9.	58; F	G	41	I	2	0/4	0/3	0/0
			43	III	3	0/4	6/6	0/8
			45	II	2	0/0	3/3	0/2
10.	49; F	G	14	III	3	0/3	nt	0/9
11.	31; M	G	23	II	2	0/3	nt	0/3
			24	II	1	0/3	6/3	0/6
12.	47; M	G	22	III	2	6/0	4/3	0/0
			11	III	3	0/6	6/6	0/6
			12	III	3	0/0	0/3	6/6
13.	53; F	G	22	III	3	0/3	nt	0/6
14.	54; F	G	13	III	3	0/3	8/8	0/3
			14	III	3	0/9	0/3	0/6
15.	56; M	G	21	III	2	0/6	nt	0/3
			11	III	2	0/3	0/3	0/3
			23	II	1	0/3	3/0	0/3
16.	43; F	L	17	II	3	0/4	4/0	nt
17.	32; M	L	33	II	3	0/8	3/0	0/2
			41	II	3	0/2	0/2	0/0
			42	II	3	0/12	6/6	0/0
18.	43; F	G	21	II	2	0/3	nt	0/6
			27	III	3	0/6	6/3	0/3
			25	III	3	0/6	nt	0/8
19.	59; F	L	17	II	2	0/4	4/0	0/3

Table 1 cont.

Patient	Age (years); sex	CP form	Proximity of tooth no.	CAL	Grading	Bcl-2*	p53*	caspase-3*
20.	43; M	L	11	III	3	0/9	0/9	0/12
			15	III	3	nt	6/0	0/3
			23	I	2	0/4	6/3	0/3
21.	29; M	L	32	II	2	0/6	0/0	0/6
			42	II	3	0/9	nt	0/3
22.	47; F	G	31	III	2	0/0	nt	0/0
			41	III	3	0/0	0/2	0/0
23.	39; F	L	31	II	3	0/3	nt	0/3
			42	III	2	0/12	2/0	0/0
24.	50; F	G	13	III	2	0/0	6/3	0/3
25.	53; F	L	31	III	3	0/0	0/3	6/3
			41	III	2	0/6	0/3	0/2
26.	51; M	G	16	I	1	0/4	0/3	0/3
27.	28; M	L	21	III	3	0/6	0/2	0/9
			12	III	3	0/8	nt	9/0
28.	21; F	L	13	II	2	3/4	4/0	0/6
29.	58; M	G	31	III	0	0/0	nt	0/3
30.	56; F	G	15	III	3	0/12	3/3	0/2

*F – Female; M – male; G – generalised type of CP; L – localized type of CP; nt – not tested; * – expression of apoptosis proteins in 12 point semi-quantitative IRS scale (see Material and Methods); the first number indicates intraepithelial localisation, the second in lamina propria ; CAL – clinical attachment level/loss*

gery, uncovering of the retained teeth and/or dental extraction. The tissue material related to the studied group (CP) and control group with respective clinical data was obtained from the Private Dental Practice in Ostrów Wlkp (MH). Written informed consent was obtained from every patient before gingivectomy, and approval for the study was granted by the institution's Ethical Committee (no 695/05). The entire tissue material was fixed for 24 hours in 4% buffered formalin and, then, embedded in paraffin. The mean size of the tissue material was 4x2x2mm. Serial 5μm thick sections were deposited onto SuperFrost/Plus microscope glasses.

Table 2. Semiquantitative analysis of tissue expression of apoptosis protein in control group

Patient/age (years), sex	Bcl-2*	p53*	Caspase-3*
1. 29; F	0/0	0/0	0/0
2. 15; M	0/0	3/0	6/0
3. 24; F	0/3	3/3	9/0
4. 65; M	0/6	0/0	2/0
5. 45; F	0/0	4/2	9/0
6. 36; M	0/4	0/0	3/0
7. 25; F	0/0	2/2	0/6
8. 36; F	0/0	0/0	3/0
9. 24; F	2/0	0/2	nt
10. 21; F	0/3	3/3	6/8
11. 29; F	0/3	6/6	6/6
12. 28; M	0/3	3/3	6/0
13. 23; F	0/3	6/2	3/0
14. 16; M	0/3	0/2	3/0
15. 16; M	0/0	Nt	3/0

*F – female; M – male; nt – not tested; * – expression of apoptosis proteins in 12 point semiquantitative IRS scale (see Material and Methods); the first number indicates intraepithelial localisation, the second in lamina propria*

Detection and studies on cellular localisation of Bcl-2, p53 and active caspase-3 in gingival samples took advantage of the classic ABC (streptavidin-biotinylated peroxidase complex) immunocytochemical technique with LSAB methodology (LSAB+ System HRP, Dako, Glostrup, Denmark), described earlier [25]. Mouse or rabbit anti-human monoclonal antibodies (mAbs) were employed, directed against Bcl-2 protein (ready to use) (Dako) and p53 protein (in dilution 1:50) (Dako) and against active form of caspase-3 protein (in dilution of 1:50) (R&D Systems). The sections were incubated with these primary mAbs at night at 4^{0}C, and afterwards were incubated with the LSAB+ System HRP (Dako, Glostrup, Denmark). Following deparaffination and rehydration, the preparations were additionally boiled in 10mM citrate buffer in a 700 W microwave oven for 18 min (in case of anti-Bcl-2 and anti-p53), washed in PBS and, then, subjected to the reaction according to the standard procedure. Every test was accompanied by a negative control, in which specific antibodies were supplemented by a normal serum of a respective spe-

cies in 0.05M Tris-HCl, pH~7.6, supplemented with 0.1% bovine serum albumin (BSA) and 15mM sodium azide.

The results were evaluated using semi-quantitative 12-points immunoreactive score (IRS) scale [26]. The preparations were examined under a light microscope, at 400x magnification. In each section of gingiva, five fields in epithelium and five of *lamina propria* were examined.

Statistical analysis

The descriptive statistics included calculation of mean, mediane values, standard deviation and standard error of the mean for quantitative traits. For qualitative variables, a distribution of patient numbers in individual categories of a trait was analysed.

Statistical analysis took advantage of the Mann-Whitney U test for nonparametric independent data and the Wilcoxon test for nonparametric dependent data. Correlations between data rows were determined employing Spearman's rank correlation index.

For comparisons of three independent samples the nonparametric Kruskal-Wallis test was used. Test for two structural indices was used to evaluate differences between groups related to percentage detectability of selected traits.

Probability values less than 0.05 were considered significant.

Results

Principal clinical data and intensity of expression manifested by apoptotic proteins in gingival samples of CP patients and control individuals are presented in Tables 1 and 2. In tissue material (68 gingival fragments) of CP patients the highest inflammatory activity (grading 2 and 3, G2, G3) was detected in, respectively, 24 (G2) and 36 gingival fragments (G3) (Table 1).

Expression of the anti-apoptotic Bcl-2 protein in patients with CP

Tissue expression of the protein was demonstrated in gingival samples of 90% patients with CP and it was significantly more pronounced than in the control (60%) (Figure 1). In semi-quantitative evaluation of Bcl-2 expression also significantly higher amounts of the protein were detected in patients with CP (4.75 ± 0.41) than in the control (2.00 ± 0.49) (Fig-

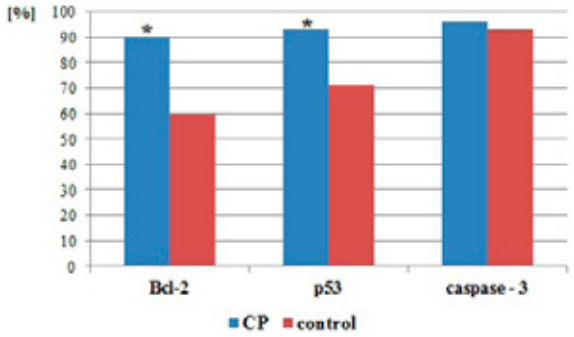

Figure 1. Comparison of detectability of apoptotic proteins (%) in gingival samples of the patients with chronic periodontitis (CP) and in the control; *p < 0.05

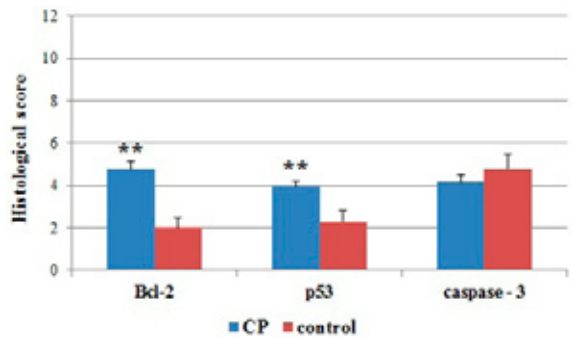

Figure 2. Comparison of tissue expression of apoptotic proteins (12-points IRS scale) in gingival samples of the patients with chronic periodontitis (CP) and in the control; **p < 0.01

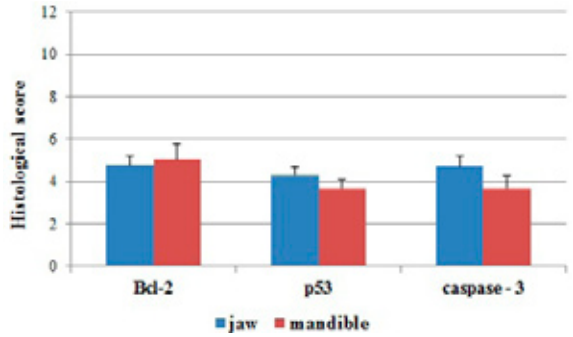

Figure 3. Comparison of tissue expression of apoptotic proteins (IRS scale) in gingival samples between maxillary and mandibular localisation of chronic periodontitis

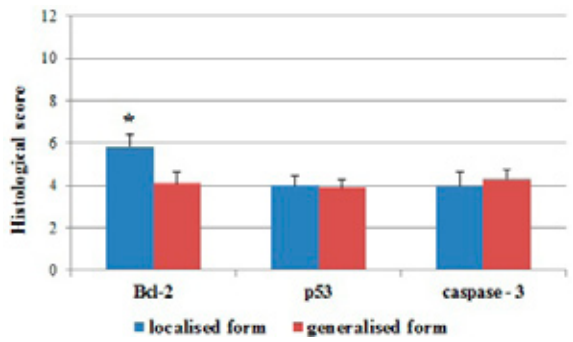

Figure 4. Comparison of tissue expression of apoptotic proteins (IRS scale) in gingival samples between localised and generalised form of chronic periodontitis

ure 2). No quantitative differences were disclosed in Bcl-2 expression which would be related to the location of the tooth (maxilla vs mandible) (4.76 ± 0.45 vs 5.04 ± 0.74) (Figure 3). A more pronounced expression of Bcl-2 protein was observed in the localised form of CP (5.83 ± 0.62) than in its generalised form (4.10 ± 0.51) (Figure 4).

Product of IHC reaction was present mainly in perinuclear space of numerous mononuclear cells of inflammatory infiltrates (mainly lymphocytes) (Figure 5A) and, sporadically, in the cell nuclei themselves. Significantly more cells with expression of Bcl-2 were demonstrated in cells located in *lamina propria* (4.59 ± 0.41) as compared to epithelial cells (0.42 ± 0.16) (Figure 6).

Analysis of proapoptotic protein expression in patients with CP

p53 protein

The protein was detected in samples of 93% patients with CP and in 71% control patients, which involved a statistically significant difference (Figure 1). Semi-quantitative analysis demonstrated significantly higher amounts of p53 protein in patients with CP (3.93 ± 0.28) as compared to control patients (2.28 ± 0.53) (Figure 2). On the other hand, no significant differences in expression of the protein were detected between maxilla and mandible (4.30 ± 0.40 and 3.67 ± 0.43, respectively) (Figure 3) or depending upon the form of the disease (localised or generalised form) (4.00 ± 0.47 vs 3.89 ± 0.35) (Figure 4).

p53 protein was detected in cell nuclei in cells of inflammatory infiltrate (mainly lymphocytes) and in keratinocytes of gingival epithelium and/or of periodontal pocket (Figure 5B). Individual cells of inflammatory infiltrates with cytoplasmic expression of p53 protein were found to be enlarged (macrophages, dendritic cells). No quantitative differences could be demonstrated in cellular location of the protein (*lamina propria* vs keratinocytes of epithelium) (2.59 ± 0.30 vs 2.88 ± 0.38) (Figure 6).

Caspase-3

Detectability of the protein in CP patients amounted to 96% and manifested no significant difference as compared to the control (93%) (Figure 1). Surprisingly, no quantitative differences between CP patients

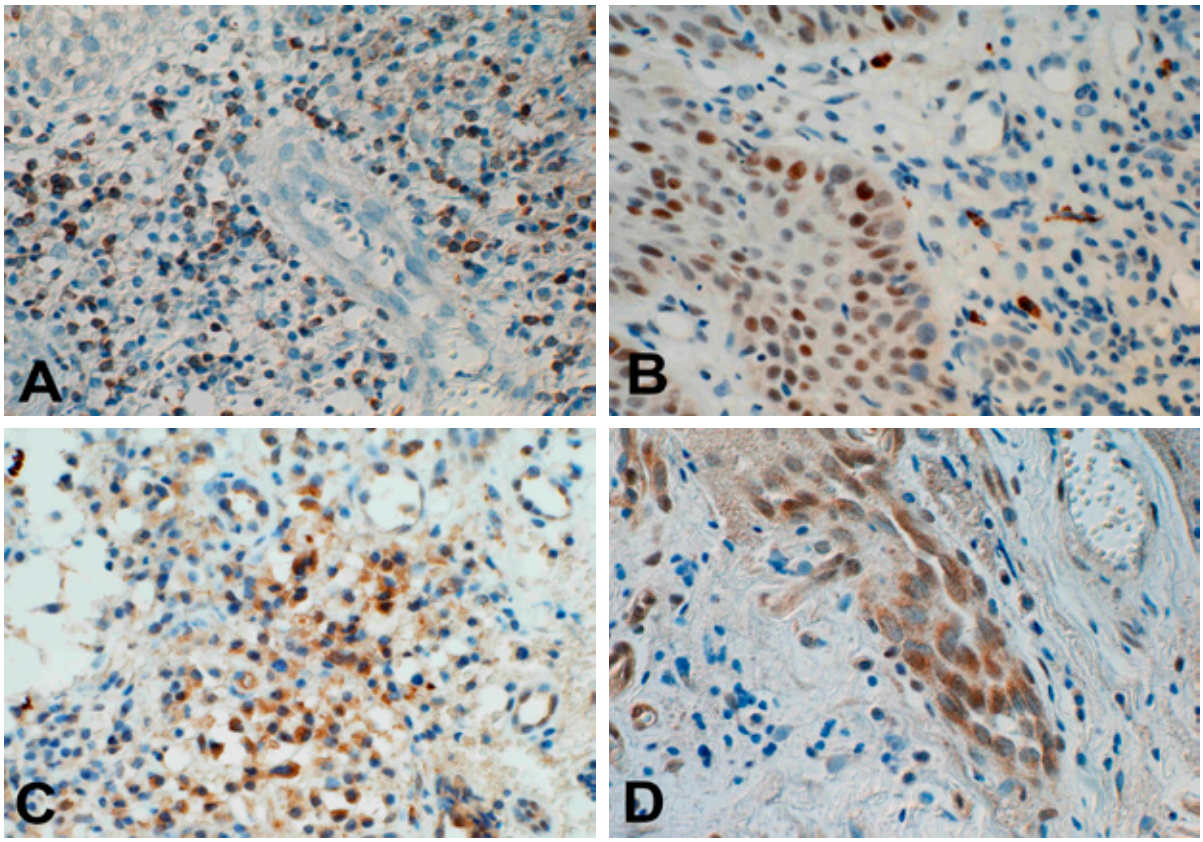

Figure 5. Immunocytochemical localisation of apoptotic proteins in gingival samples of the patients with chronic periodontitis. (A) perinuclear/nuclear localisation of Bcl-2 protein in inflammatory cells in *lamina propria;* (B) nuclear (in keratinocytes) and cytoplasmic (mostly in *lamina propria* cells) localisation of p53 protein; (C) cytoplasmic localisation of caspase-3 in inflammatory cells in *lamina propria;* (D) cytoplasmic localisation of caspase-3 mostly in keratinocytes of gingival sample. ABC technique. Hematoxylin counterstained. Objective magnification x40

(4.17 ± 0.36) and the control (4.78 ± 0.73) were detected also in expression of caspase-3 (Figure 2). In contrast to expectations no significant differences in expression of the protein of effector phase of apoptosis were noted between generalised form of CP (4.30 ± 0.42) and its localised form (3.96 ± 0.68) (Figure 4), nor between maxillary and mandibular localisation (4.70 ± 0.49 and 3.68 ± 0.58, respectively) (Figure 3). As compared to epithelial keratinocytes (0.62 ± 0.23), a significantly higher expression of the protein was detected in cells of inflammatory infiltrate (mainly lymphocytes) in *lamina propria* (4.03 ± 0.36) (Figure 6).

Microscope examination demonstrated presence of caspase-3 in cell cytoplasm of cells in inflammatory infiltrates, epithelial cells, individual cells of vascular endothelium and fibroblasts (Figure 5C and D). Very frequently, the IHC reaction was very intense.

Reciprocal correlations between expression of apoptosis proteins in gingival samples of CP patients

No significant relationships were detected between reciprocal expression of individual apoptosis proteins ($p > 0.05$) in patients with CP (Table 3).

Reciprocal correlations between expression of apoptosis proteins in control gingival samples

In control group the only significant and direct relationship was detected between expression of two pro-apoptotic proteins (p53 and caspase-3) ($r = 0.547$; $p < 0.05$) (data not shown).

Expression of apoptosis proteins in gingival samples of CP patients and selected clinical data

In tissue material obtained from patients with CP no significant differences were detected in intensity of expression of apoptosis proteins which would depend on activity of inflammatory lesions (*grading)* or degree of CAL (Tables 4 and 5).

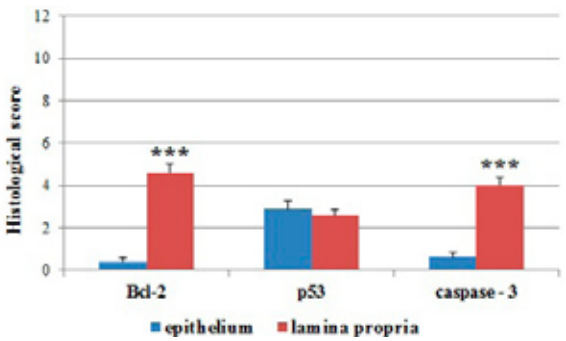

Figure 6. Comparison of tissue expression of apoptotic proteins (IRS scale) in epithelial cells and *lamina propria* cells in gingival samples of the CP patients; ***$p < 0.001$

Table 3. Coefficients of Spearman's correlation between reciprocal tissue expression of apoptosis proteins within the patients with chronic periodontitis

	r	p
Expressions of Bcl-2 and p53	0.012	0.934
Expressions of Bcl-2 and caspase-3	0.049	0.705
Expressions of p53 and caspase-3	0.216	0.140

r – Spearman's correlation coefficient; p – level of significance

Table 4. Comparison of expression manifested by apoptosis proteins depending on intensity of inflammatory lesions (grading) in gingival tissues with chronic periodontitis (Kruskal-Wallis test)

	p
Expression of Bcl-2 and grading	0.067
Expression of p53 and grading	0.168
Expression of caspase-3 and grading	0.229

p – level of significance

Table 5. Comparison of expression of apoptosis proteins depending on clinical attachment level/loss (CAL) in gingival tissues with chronic periodontitis (Kruskal-Wallis test).

	p
Expression of Bcl-2 and CAL	0.427
Expression of p53 and CAL	0.549
Expression of caspase-3 and CAL	0.305

CAL – clinical attachment level/loss; p – level of significance

Discussion

Bcl-2 is a protein of 25 kDa molecular mass and is involved in control of cell programmed death. Expression or overexpression of the protein may protect the cell from death, preventing or delaying apoptotic process [27]. Both detectability of Bcl-2 in patients with CP and its expression intensity have been significantly higher in patients with CP than in healthy gingival tissues, which is consistent with results obtained by other authors in case of generalised aggressive periodontitis (AP) [28]. Our studies have demonstrated slightly higher amounts of Bcl-2 protein in the localised form of CP as compared to the generalised form of CP. Microscopic analysis has documented presence of IHC reaction product in numerous mononuclear cells of inflammatory infiltrate (mainly lymphocytes) within *lamina propria*. On the other hand, poor expression of the protein in lymphocytes of inflammatory infiltrate in CP was shown by Sawa et al. [21]. Our studies have confirmed the perinuclear location of Bcl-2 protein [29]. In cultured human gingival fibroblasts infected with *P. gingivalis* activation of Bcl-2 was observed in early stages of the infection (between second and 12th hour)

[18]. Other investigations demonstrated an increase in Bcl-2 expression and inhibition of apoptosis by *P. gingivalis* also in cells of gingival epithelium but failed to described time-dependent alterations in expression of the protein [22]. Carvalho-Filho et al. hypothesize that *P. gingivalis* HmuY protein plays a role in the pathogenesis of CP, possibly by reducing or delaying apoptosis in T cells through a pathway involving the Bcl-2 protein [30].

The higher expression of Bcl-2 anti-apoptotic protein detected in gingival samples (in addition in cells of *lamina propria)* of our patients with CP than in control group may suggest inhibition of apoptosis in cells of inflammatory infiltrate (mainly lymphocytes), their longer survival and, as the result, their destructive effect of periodontal tissues. However, we have failed to note significant relationships between presence of Bcl-2 and clinical indices such as grading and CAL values. Possibly, this might have reflected prevalence in the material of highly intense inflammatory lesions (grading 3) and high values of CAL parameter (CAL III) in most of the sampled fragments of gingiva with CP. On the other hand, a significantly higher expression of Bcl-2 protein has been detected in the localised as compared to generalised form of CP, which would suggest that the former form of CP is accompanied by a higher proportion of cells in inflammatory infiltrate with an extended life span (extended inhibition of apoptosis).

p53 protein, of 53 kDa molecular mass, termed the genome guardian, is responsible for DNA repair and, if the lesions cannot be repaired, for induction of programmed death of the cell [31]. It leads to apoptosis of terminally differentiated cells, including cells of inflammatory infiltrate [28]. IHC techniques permit to detect only the protein of an elongated half-life (a mutated one or manifesting an increased synthesis coefficient) [31].

In this study p53 protein has been detected mostly in cell nuclei, both in cells of inflammatory infiltrate (mostly lymphocytes) and in keratinocytes. Detection of the protein both in the tissue material from patients with CP and in the control may point to activation of p53 production under effect of stress factors (*e.g.*, inflammation, permanent exposure to bacterial infection in oral cavity). The more pronounced expression of p53 in patients with CP than in control individuals may indicate potential role of the protein in initiation of apoptosis (at least in some cells of inflammatory infiltrate) or may provide proof for an augmented reparative role of cell nucleus structures (mainly DNA), injured due to chronic inflammation. Other authors could not demonstrate quantitative differences in expression of the protein between patients with aggressive periodontitis and control individuals [28]. The data of Ghosh et al. demonstrated that a proapoptotic fibronectin matrix induces the degradation of ubiquitinated p53 *via* proteasomes in periodontal ligament cells. The authors concluded that is may be a novel mechanism of apoptosis associated with inflammatory diseases [32]. In this study we have not been able to demonstrate a relationship between expression of p53 protein and form of the disease or remaining clinical data.

Caspases represent a family of cysteine proteases which enzymatically split various substrates contain in the cell [33]. Activation of caspase-3 starts the effector apoptosis phase, leading to a cascade of event terminated by cell death and its elimination. Detection in a tissue of cells immunopositive for the so called active caspase-3 form is thought to be a significant trait of apoptosis activation [34]. It is suggested that activation of caspases represents the main character of tissue injury in the course of periodontitis. In tissues of patients with CP activation of caspase-3, caspase-7 and splitting PARP, the caspase-3 substrate was demonstrated both in keratinocytes and in connective tissue cells [10]. According to the authors, detection of high number of cells containing active forms of caspases proves that early stages of apoptosis are detected.

In our study the cytoplasmic product of IHC reaction for caspase-3 has been detected in cytoplasm of inflammatory infiltrate cells and in epithelium of gingiva and/or periodontal pocket. A significantly higher expression has been noted in cells of inflammatory infiltrate (mainly lymphocytes) than in keratinocytes. No differences have been detected in detectability and intensity of caspase-3 expression between tissues obtained from CP patients and control individuals, consistent with the data of Bantel et al. [10]. A similar absence of differences was demonstrated in cases of AP and control [28]. Other studies demonstrated that fibroblasts represent the principal apoptosis-affected cell population in CP, apart from only small proportion of leukocytes. The authors suggested that apoptosis of fibroblasts represented a normal phenomenon in cases of remodelling of tissues such as periodontium and that it indicated presence of an equilibrium between fibroblast proliferation and their apoptosis [24]. The results of Lucas et al. indicated that apoptosis in periodontitis may be inhibited by elevated expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) decoy receptors and cleaved caspase-3 inhibitors [35].

In contrast to expectations, our investigations have failed to demonstrate significant differences in expression of effector phase apoptotic proteins between the localised and generalised forms of CP. Both detectability of the protein in patients with CP and intensity of the reaction (semiquantitative evaluation) have not significantly differed from the control. Gamonal et al. demonstrated cells with active caspase-3 and other apoptotic proteins only in patients with chronic periodontitis [8]. In their studies numerous cells in apoptosis were detected also in biopsies of gingival samples with PD⩾5 mm and CAL⩾3 mm. The principal cells in apoptosis involved neutrophils even if the process affected also other cells of inflammatory infiltrate [8].

In our studies we have not been able to document significant relationships between expression of caspase-3 and clinical data. Also, no reciprocal correlations have been observed in expression of apoptosis proteins within the group of patients with CP.

Results of our investigations on apoptosis markers in gingival cells of clinically healthy periodontium (lower expression of Bcl-2 and p53, but similar expression of caspase-3 as compared to CP patients, positive correlation between the two pro-apoptotic proteins) is consistent with the suggestion that periodontium tissues, continuously exposed to low degree bacterial infections may contain cells with DNA damage and a variable expression of apoptotic proteins. Tonetti et al. examining control gingival samples demonstrated apoptotic cells in particularly high numbers in subepithelial clumps of inflammatory infiltrates and within joining epithelium [36]. The caspase-3 dependent mechanism of apoptosis in experimental periodontitis in diabetic rats inoculated with *A. actinomycetemcomitans* showed increasing the inflammatory response with enhancing apoptosis of gingival epithelial and connective tissue cells [37].

Summary and conclusions

1. In chronic periodontitis significant alterations develop in expression of immunocytochemical exponents of apoptosis, with prevalence of Bcl-2 and p53 proteins, as compared to the control.
2. The localised but not the generalised form of CP is accompanied by higher proportion of Bcl-2 positive cells of inflammatory infiltrates, which suggests inhibition of apoptosis in this form of chronic periodontitis.
3. A comparable expression of caspase-3 (effector phase of apoptosis) in samples of CP patients and in clinically healthy periodontium and absence of correlation with advancement of clinical lesions in chronic periodontitis suggests that apoptotic process plays no significant role in destruction of periodontal tissue in CP.

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REVIEW PAPER

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A brief history of taxol

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ABSTRACT

Traditional application of plant in folk medicine is a base for discovery of new active chemical substances. In the second half of 20th century, significant antitumor activity of extract from bark of the Yew tree was recognized. The compound that was responsible for this kind of properties has called taxol and had structure of diterpene with acyl groups. Low availability forced researchers to find different ways of taxol gaining. In the further years, many synthetic and semi-synthetic methods were developed. Nowadays, biotechnological methods with use of cell suspension cultures are introduced.

Key words: cancer, taxol, chemotherapy.

For a very long time, raw plant materials were used for medical purposes [1, 2]. The knowledge considering directions and ways of their therapeutic applications was mainly based on traditional folk medicine. With development of different kinds of qualitative and quantitative chemical analysis, the knowledge of chemical composition of natural therapeutic agents was broadened. Nowadays, besides of very good development of chemical synthesis technology, raw plant materials are still use for isolating many active substances, mainly in enantiomerically pure form. Very often this kind of natural products are the starting point for new compounds synthesizing in belief, that structural analogies may be applicated to gain more effective drugs.

The one of the more interesting examples illustrating this type of attitude of searching for new, biologically active substances is a history of taxol – acylated diterpene which was isolated from yew tree bark with affirmed strong antitumor activity.

Therapeutic properties of yew tree were applicated in practice since the ancient times [3]. Then its toxic activity was also found. In XIX century, on the base on many hundred years experience of traditional folk medicine, a lot of compounds responsible for biological activity of yew tree were isolated. In sixties of XX century, cytostatic properties of extracts from yew tree bark (*Taxus brevifolia*) were developed and confirmed. The structure of active substance named taxol was publicated for the first time in 1971 [4] (Figure 1).

Initially, the only one source of this compound was *Taxus brevifolia* bark; nevertheless taxol is a metabolite formed by each species of yew trees in different but small amounts. Unfortunately, the whole procedure of isolation of this substance from raw plant material *T. brevifolia* was hardly arduous work and underwent with low yield. Isolation of 1 kg of raw natural product required the use of 10 tons of yew tree bark. It means that 3000 of trees ought to be cut out. Also, it shoud be point out, that 1 kg of taxol is sufficient for 500 patients; moreover *Taxus brevifolia* is one of the slowest growing trees in the world. The total number of this yew species in the Northwest of United States was then estimated to one million. Further exploitation of *T. brevifolia* for experimental purposes endangered liquidation to natural places of yew tree. Moreover, the obvious ecological aspect of this problem, it was automatically connected with abandon of very promising research on taxol. It was essential to elaborate some different methods – more costless and higher yielding to obtain this valuable substance from acylic diterpene taxanes group. Within the confines of searching of optimal solution, it was found that in a result of extraction of fresh *T. baccata* needles, different taxane diterpene was gained. It was 10-deacetylbaccatin, which was

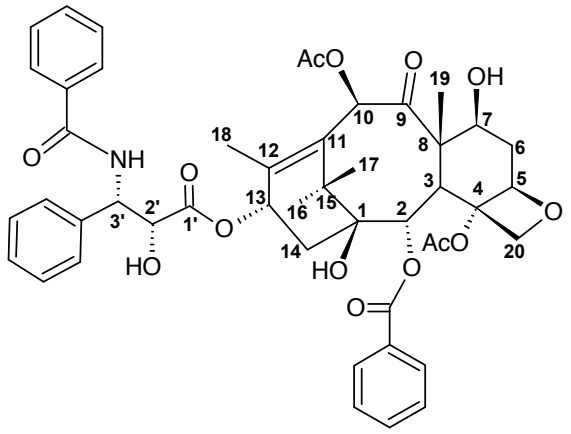


Figure 1. Structural formula of taxol with terpenic skeleton carbon atoms numeration

structurally closed to taxol [5]. The process of isolation underwent from more favourable material and with higher yield – from 3 tonnes of needles, about 1 kg of substance was obtained. Moreover, the procedure did not require destroying of yew trees, because needles are easily renewable. From the moment of elaborating an effective method of transformation of 10-deacetylbaccatin in taxol, it might be obtain in semisynthetic way. This method is based on side chain addition as a result of esterification reaction of C-13 hydroxyl group in 10-deacetylbaccatin with N-benzoylphenylisoserine. This process requires earlier selective esterification of secondary C-7 and C-10 hydroxyl groups. Because of the different reactivity of hydroxyl groups in molecule, first step concerns triethylsilylation of C-7 OH; additionally C-10 hydroxyl group has to be acetylated. This semiproduct may be coupled in reaction with enantiomeric O-protected N-benzoyl-α-hydroxy-β-aminoacid. After deprotection of hydroxyl groups with HCl, taxol is obtained with yield up to 90% (Figure 2).

For synthesis of benzoyl aminoacid system named side chain, different types of reactions were used. In the first publicated method, the Sharpless epoxidation was used as a key step. The optically pure epoxide opening reaction with azide anion gave product with expected configuration. Further steps: O-benzoylation, reduction connected with transfer of benzoyl group from oxygen atom to nitrogen atom, then protecting hydroxyl group and hydrolysis of ester group gave demanding 3-phenylisoserine derivative.

In 1994 R. Holton with co-workers presented the total synthesis of taxol [6]. (–)-Camphor was the main substrate for whole, forty-steps process. But for the reason of very low yield (0.4%) and lack of profitabil-

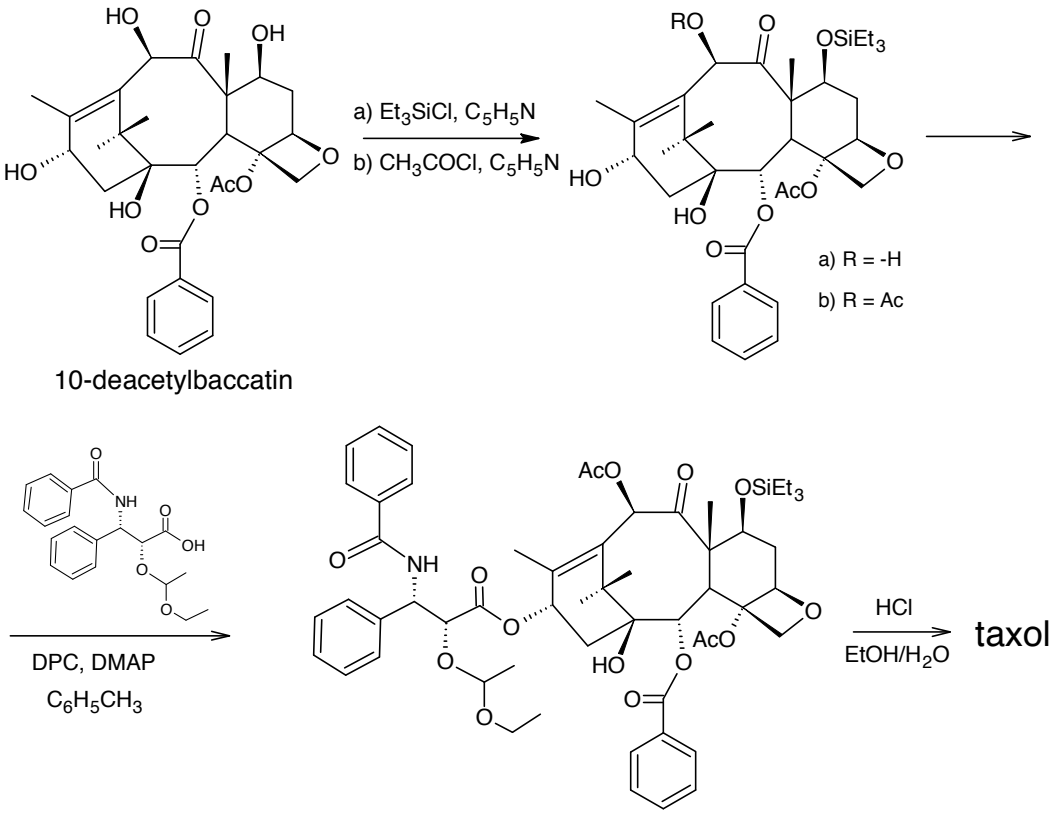


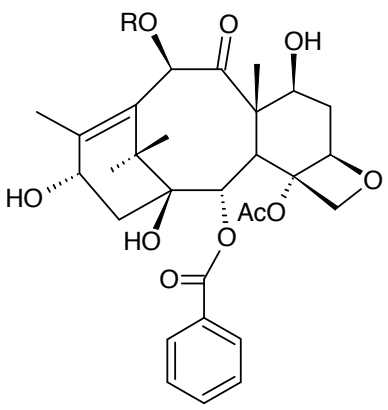
Figure 2. Semi-synthetic method of obtaining taxol from 10-deacetylbaccatine

ity, this method did not find any practical application in pharmaceutical industry. During last a dozen or so years, many reports has been appeared describing the use of *in vitro* cell suspension cultures for biosynthesis of taxol [7–10]. Cell cultures ensure continuity of supplying the starting substances and are independent on quality of raw plant material. Because of control possibility of culture conditions, it may lead to increase of bioproduction of baccatin and 10-deacetylbaccatin. Both are taxol precursors. The structures of these compounds are shown on Figure 3.

Biotransformation methods are very favourable due to the final product of this kind of synthesis is characterized by high purity. Also, the yield of this process is usually high enough. These reactions are highly specific and they let to obtain large amounts of natural compounds which often appear in small quantities in plants. Lastly, R. Venilla and J. Muthumary [8] presented the method of gaining taxol from endophytic fungi cultures *Pestalotiopsis sp*. isolated from tropical tree bark named *Tabebuia pentaphylla*. To confirm the presence of taxol, the fungal samples were subjected to HPLC method. The quantity of final product was estimated to be 208.6 µg/L. Other authors [9, 11, 12] presented the way of obtaining 10-deacetylbaccatin, taxol and other taxane derivatives with use of plant cell suspension cultures isolated from eg. eucalyptus (*Eucalyptus perriniana*), common liverwort (*Marchantia polymorpha*), Indian snakeroot (*Rauwolfia serpentina*), tobacco (*Nicotiana tabacum*) and soya bean (*Glycine max*). Nowadays, there are still some research to improve the production methods of diterpene compounds eg. with use of jatropha (*Jatropha curcas*) [7].

There are many reports describing relationships between taxol structure and its pharmacological activity (SAR) [13, 14]. The basic diterpene skeleton includes ten chiral centers and additionally C-13 side chain has two asymmetric carbon atoms. Depicted on Figure 1 stereochemical system is essential for cytostatic activity of taxol. Several publications have concerned the influence of presence of particular hydroxyl groups, that are substituents in main diterpene skeleton, on SAR. It was found, that removal of C-7 OH and acetoxyl group at C-10 position did not change significantly the cytotoxicity of molecule. However, 4-deacetyl- and 4-deacetoxytaxol had lowest antitumor activity. Also, 1-deoxytaxol was 10-fold less active in comparison with taxol. Substitution in para- position at 2-benzoyl group also decreased the level of biological activity. Interestingly, analogs with ortho- and particularly meta- substituted aroyl substituents were usually more active than taxol. Some of them were even sixfold more cytotoxic. The summary of taxol SAR is presented in Figure 4.

Calculated partition coefficient value (ClogP) for taxol molecule is 4.73. It indicates on high lipophilicity of this compound. Its aqueous solubility is less than 0.01 mg/L, so this substance is practically insoluble in water. The solubility difficulties made its application to patients very limited. This problem was tried to be dissolved in several ways. At the beggining, taxol was administered as a 6 mg/mL surfactant – Cremophor EL and ethanol mixture diluted with normal saline or 5% dextrose in water to the required final concentration [15]. Because of the relatively high taxol doses, patients also received large doses of surfactant, which was not neutral for people health. Several years ago, nanotechnology gave an effective possibility of supplying highly hydrophobic drug to the organism [16, 17]. With use of nanotechnology, methods were elaborated concering incorporation of active agent, insoluble in water, into biocompatible nanomolecules that



baccatin: R= Ac

10-deacetylbaccatin: R= -H

Figure 3. Structure of baccatine and 10-deacetylbaccatine

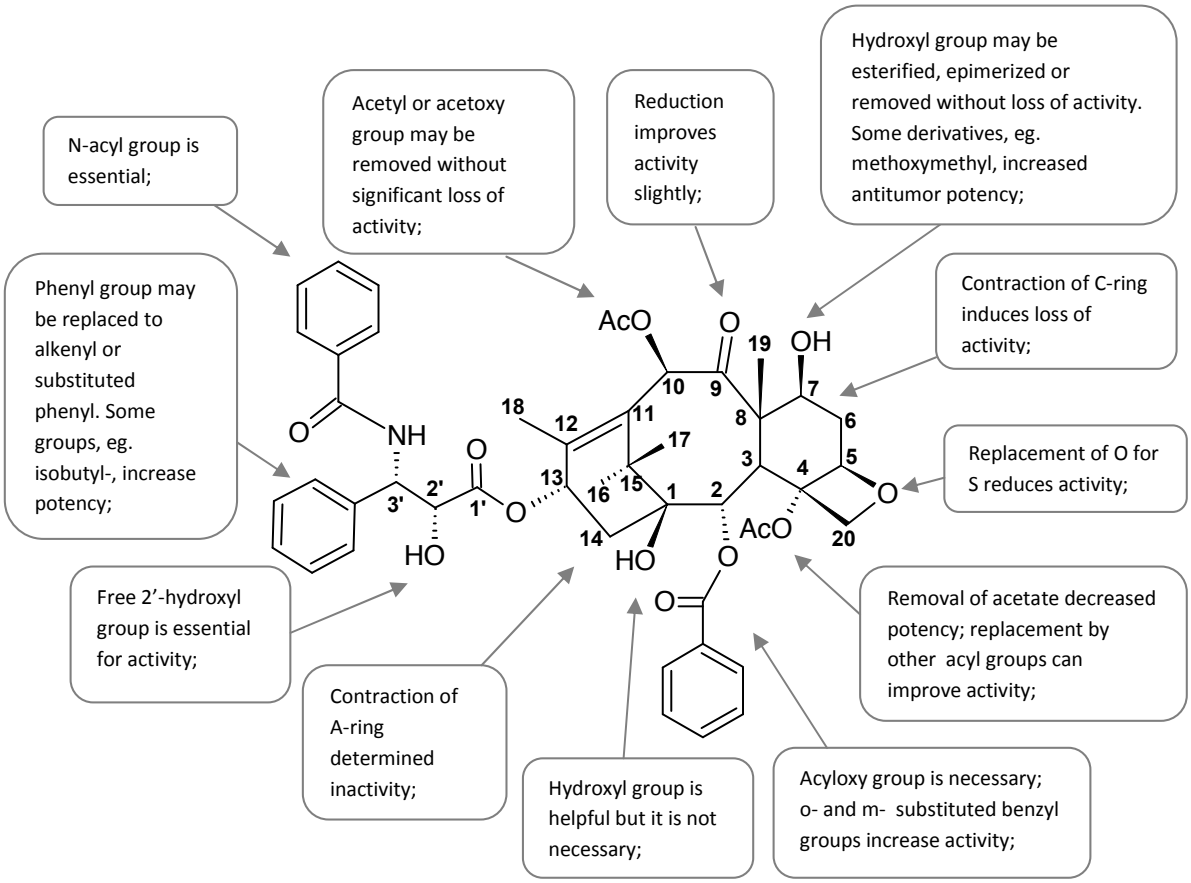


Figure 4. Structure-activity relationships of taxol

were obtained on the base of silica [18] or functionalized carbon [19]. Some others named block copolymer micelles have structures as core – shell type. They have capacity to transport large therapeutic payloads and passively target tumor sites [20]. Their active surface area is about 1000 m^2/g, that is why block copolymer micelles may serve as a carrier for hydrophobic active substances. Moreover, micellar shell is hydrophilic, so these structures are highly soluble in polar medium. It allows to deliver safely and in non-invasive manner the hydrophobic drug to organism cells [21]. In case of taxol, the use of nanotechnology method had additional advantage. Because of specific targeting of nanomolecules, the retention time was prolonged and accumulation of active substance in tumor tissue was increased. What is important, the healthy biological systems were not destroyed [22]. Another way of improving bioavailability of hydrophobic molecule is making some modifications of functional groups and obtaining derivatives with better solubility in aqueous media and with comparable or improved range of therapeutic activity. After making chemical modifications at the end fragment of C-13 side chain and deprotecting C-10 hydroxyl group, a new taxol derivative was obtained. This substance named taxotere [23]. It has t-butoxycarbonyl moiety instead of benzoyl fragment on the amino group of phenylisoserine chain at C-13 and a hydroxyl group instead of acetoxy group at C-10, as a result of selective hydrolysis reaction (Figure 5).

Favourable synthesis of taxotere, its high antitumor activity and relatively good way of application contributed to further development of experiments, directed to obtain even more effective taxol modification products. A series of taxol esters were tested. They were gain as a result of hydrogen atom substitution in OH group at C-2' in side chain and in OH group at C-7 of main diterpene skeleton with aminoacid aryl rests [24]. After biological examination of obtained derivatives, it was found that substitution of C-2' hydroxyl group lead to compounds with comparable pharmacological properties with taxol. It is connected with higher susceptibility of this group on the hydrolysis reaction under *in vivo* conditions and transformation of these esters into starting diterpene or its active metabolites.

Both taxol and taxotere are substances registered as therapeutic agents, named respectively as Paclitaxel

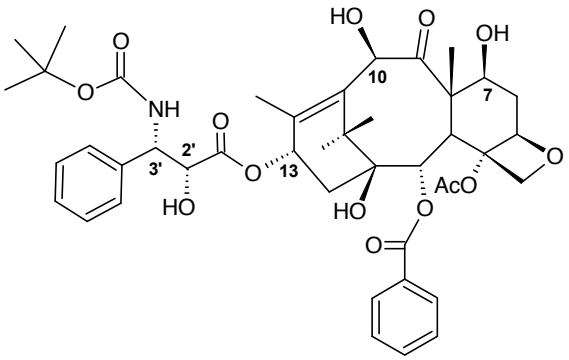
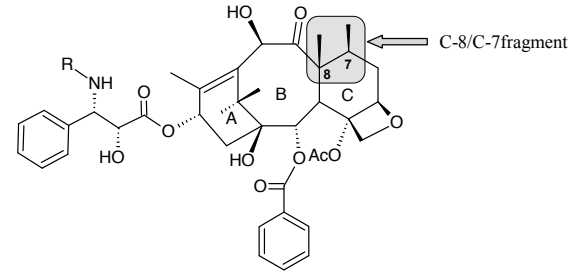


Figure 5. The structure of taxotere



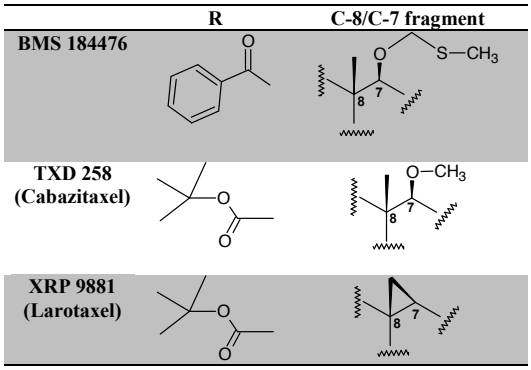


Figure 6. Structure of active, second generation diterpenes

and Docetaxel. Mechanism of action for both drugs consist in stabilization of microtubules in cell, which counteract their depolimerization [25]. This process inhibits correct separation of two identical sets of chromosomes and their transfer during cell division. Docetaxel and Paclitaxel cause blockage of cell mitosis and consequently induce cell death.

Nowadays, several different compounds of second generation from taxol diterpene group undergoing the sequence of clinical trials. Among them are BMS-184476 and XRP-9881 (Larotaxel) [26]. Moreover TXD-258 has finished successfully clinical trials and has been registered as Cabazitaxel [27] (Figure 6). It can be used in therapy of some tumors that are resistant to Docetaxel.

Conclusion

Studies on taxol are conducted broadly since 1967, so practically since the moment of it development. The methods of taxol obtaining are still improved. Also, there are some effects in fighting against aqueous solubility limits. Each year, there is more knowledge about it mechanism of action and structure – activity relationships that have influence on pharmacological activity of its derivatives. It is necessary to be hopeful that these factors will contribute to further development of antitumor chemiotherapy and will be fruitful in the future in discovering a series of new, even more potent substances in this group.

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REVIEW PAPER

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Sympathetic Nervous System activity – a new concept of the complicated etiology of low back pain radiates distally at the extremities

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ABSTRACT

Varied and complicated etiology of low back pain radiates distally at the extremities is still causing disagreement and controversies around the issue of its diagnosis and treatment. New research data demonstrated that almost one in five persons with back pain experience symptoms indicative of neuropathic pain component. The neuropathic involvement is not completely understood, and different mechanisms are thought to play important role. A combination of nociceptive and neuropathic pain-generating mechanism is thought to be involved, which established the term mixed pain syndrome. In the pathomechanism of neuropathic pain the lesion, trauma or overloading of the disc is thought to be a primary source of the neuropathic pain but the concept of neuropathic component of pain is more probable for chronic stage than acute. Assessment of neuropathic pain involves a systematic approach which includes a series steps; past and present history, detailed description of pain distribution, quality, pain intensity and neurological examination with emphasis on sensory testing. The sensory examinations need often to be supply neurophysiological testing and quantitate sensory testing. Some groups of the drugs are thought to be useful e.g. tricyclic antidepressant, sodium channel blockers (e.g. carbamazepine), gabapentin, opioids, NMDA (N-methyl-D-aspartate) receptor blockers and others for neuropathic pain treatment. The use of specific kind of the drugs depends on the symptoms and examinations findings.

Key words: sciatica, neuropathic pain, low back pain, quantitative sensory testing.

Varied and complicated etiology of low back pain radiates distally at the extremities is still causing disagreement and controversies around the issue of its diagnosis and treatment. Most of the clinicians are thought that the source of that pain is generally radicular. Some of them postulated the clinical meaning of the sacroiliac joint syndrome which demands injections of lignocaine to the area of that joint for pain release. Lastly, some of the scientist postulated the new concept in the understanding "patients with sciatica". That group of the patients is clinically divided into two sub-groups, namely radicular or pseudoradicular problems [1].

It is widely accepted that acute low back pain is caused by degeneration of intervertebral discs (hernia, bulging). While for acute condition protrusion is stated as the chief reason, for a group in chronic state (approx 10–40%) lateral or foraminal stenosis or tumors [2] and neuropathic component lastly are suggested. One should remember that in the pathomechanism of neuropathic pain the lesion, trauma or overloading of the disc is thought to be a primary source of that pain [3].

New research data from Germany (2009), demonstrated that almost one in five persons with back pain experience symptoms indicative of neuropathic pain component [4]. The neuropathic involvement is not completely understood, and different mechanisms are thought to play important role. A combination of nociceptive and neuropathic pain-generating mechanism is thought to be involved, which established the term mixed pain syndrome [4]. It is indicated that neuropathic pain can occurs via mechanical nerve root compression (mechanical neuropathic root pain), lesions of

nociceptive sprouts within the degenerated disc (local neuropathic pain), or by action of inflammatory mediators such as chemokine's and cytokines, which can originate from the degenerative disc even without any mechanical stress (inflammatory neuropathic root pain) [1]. The clinical symptoms among those two groups of the patients are very similar. It is difficult to carry out full diagnostics of the above mentioned symptoms due to lack of exactly defined golden standards [5, 6].

Nevertheless, the precise examinations can help to diagnose the sympathetic nervous system involvement in the pain thought to be "radicular". The diagnosis of the radicular character of pain thought to be sciatica is done on the basis of clinical examinations, interpretation of the magnetic resonance imagination (MRI) and sometimes electromyography.

It is widely known that, the most common cause of radicular pain in the lower limb is inflammation following nerve compression caused by, for instance, disc herniation, lateral or foraminal stenosis, spondylolisthesis or tumor [7, 8–11]. Due to this fact, patients with low back pain radiates distally at the extremities with positive Lasegue's test and disc hernation confimed by MRI on the level of five and forth lumbar level are often diagnosed as a radicular pain.

However, some studies have shown poor correlation between radiological imagining and clinical symptoms [12]. What is more, in some asymptomatic persons herniated nucleus pulposus was confirmed by MRI (21% for 20–39 years of age, 22% for 40–60 years of age and 36% for above 60 years of age). Taking into consideration disc bulging even higher numbers were obtained for these age groups, respectively 56%, 59% and 79% [13]. There are also reports on patients suffering from confirmed disk pathology or with stenosis with apparent neural compromise i.e. asymptomatic [13–15]. Nevertheless, during MRI evaluation one should consider that in majority of patients with pathology within disc area a strong correlation with pain in the lower limb is visible [16], but sometimes it is possible to observe improvement with no change concerning the disk [17], or the other way round: no improvement in spite of removing the disc protrusion or other reasons of nerve compression [11]. Takashasi et al [11] claim that compression itself causes only loss of function rather than pain, which was firstly postulated by Kelly [8]. It is suggested that processes other than compression are engaged in the development of sciatica and the leading role of inflammation in causing the feeling of strong pain along the sciatic nerve is underlined [9, 18].

In the context of the paper by Freynhagen et al. [1], the above data raise even more doubts as far as the interpretation of MRI is concerned, all the more as in the pathomechanism of neuropathic pain in chronic patients the primary injury of the intervertebral disc, for instance, has been described.

Another test to confirm inflammation of the sciatic nerve is electromyography (EMG). Neurophysiological examinations to support a proximal nerve root lesion include the distal motor latency and the F-wave latency or nerves, which receive their nerve fibers from the affected root. This examination will only show pathological values if motor fibers are involved in the damage. It is important to known that the proximal lesion to the dorsal root ganglion during examinations can give norm of sensory conduction. In general, when we consider the involvement of neuropathic component of pain it is important to realize that conventional electrophysiological techniques assess only the function of myelinated peripheral axonal system [4]. The involvement of the small fibers (neuropathic pain) is possible to assess by Quantitative sensory testing (QST). That system allows to complete assessment of all sensory submodalities, including the large (Aβ) and small (Aδ and C) fibers. Unfortunately, that system is quit new and used in sciences laboratories mainly. What are more QST results should not be the sole criteria utilized to diagnose structural pathology, of either a peripheral or central nervous system (CNS) origin. Abnormalities on QST must be interpreted in the context of a thorough neurologic examination and other appropriate testing such as the EMG, nerve biopsy, skin biopsy, or appropriate imaging studies.

The next of step of the standard diagnosis of radicular pain is bedside examinations on the basis of clinical criteria e.g. positive Lasegue's test, motor sensory, or reflex deficits, apart of MRI value analysis. For many years it was believed that those clinical criteria were specific for radicular pain only. In the study of Freynhagen et al. [1] those common used criteria were confirmed among patients with pseudoradicular pain as well. That situation blurring clinical pictures of those patients and the appropriate distinguishing of CNS involvement is difficult for less experienced physician. On the basis on the new data and the previous findings about disc protrusion importance, some authors postulated that pseudoradiculopathy and radiculopathy is rather a disease continuum, than the different disease entities [4, 18].

Some explanation is needed about the diagnostic value of the Lasegue's test because of the clinical

common use of them. According to scientist the diagnostic accuracy of the neurological signs and tests is unclear [19]. The Lasegue's test is a widely used diagnostic tool for confirming sciatica. Total clinical reliability of this test is questioned, however, as it has no identical application standards or result interpretation. It was even claimed that for diagnostic purposes negative result was more significant than the positive one [12]. Simultaneous use of the Lasegue's test together with a passive ankle dorsiflexion (Bragard's procedure) for more reliable confirmation of radicular pain is suggested [20].

Some of the authors indicate possible distortion of the result for the Lasegue's test by strong hamstrings tension [21]. During the Lasegue's test the patient's description of pain is taken into consideration, which, according to many authors, raises doubts and is not too credible a tool for Lasegue's test verification. According to Backup [22], strong tension of these muscles might simulate the inflammation of the sciatic nerve. In other papers it was proven that basing on the Lasegue's test it was not possible to differentiate between patients who were asymptomatic but had strong tension in the hamstring muscle and patients with sciatica [23]. Mechanisms leading to the increase in the tension of these muscles have not been explored so far.

The crossed Lasegue's test made the diagnosis more specific for hernia thus either crossed Lasegue's test or Bragard's procedure can be used to confirm radicular character of pain in case of positive Lasegue's test. In the cases with the negative Lasegue's test or/ with unsure interpretation of the positive one, negative MRI findings we should consider sympathetic nervous system (SNS) activity, especially in chronic stage. Apart of the QST the common used tools are questionnaire: Leeds Assessment of Neuropathic Symptoms and Signs (LANSS), Neuropathic Pain Questionnaire (NPQ), Douleur Neuropathique en 4 questions (DN4), pain DETECT [24]. Clinical examination at bedside includes: pinprick, touch, cold, heat and vibration. Pinprick sensation is assessed by the response to prinprick stimuli; touch is examined by gently stroking the involved skin area with a cotton swab, cold and warm sensation is recorded by measuring the response to a specific cold or warm thermal stimulus. Vibration is assessed by a tuning fork placed at strategic points. At present there is no consensus about the site where such activity should be measured, but it is generally agreed that this is best done in the area as control. For all types of stimuli, the response can simply be graded as: normal, decreased or increased. If the response is increased, it is classified as dysesthetic, hyperalgesic or allodynic. Assessment of neuropathic pain involves a systematic approach which includes a series steps; past and present history, detailed description of pain distribution, quality, pain intensity and neurological examination with emphasis on sensory testing. The sensory examinations needs often to be supply neurophysiological testing and quantitate sensory testing [25]. The distinguishing the radicular and neuropathic component is very important because of the completely different treatment approach. There a lot of cases suffered months or years because of the "chronic sciatica" after different failed therapies. Some groups of the drugs are thought to be useful e.g. tricyclic antidepressant, sodium channel blockers (e.g. carbamazepine), gabapentin, opioids, NMDA receptor blockers and others for neuropathic pain treatment. The use of specific kind of the drugs depends on the symptoms and examinations findings.

We should remember that now clinicians are challenged with a series of possible pathophysiological mechanism of neuropathic pain and the optimal way of the treatment is difficult due to lack of the knowledge. Additionally, excellent work in the basic science of that pain is in contrast with the limitations of inadequate random controlled trials regarding long-term pharmacologic interventions. Complex rational pharmacologic strategies for structural pathology, central pain processes, sites of medication action, and differing routes of administration are delineated [26].

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The angiotensin converting enzyme inhibitors – alternative clinical applications

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ABSTRACT

Angiotensin converting enzyme inhibitors have emerged as a useful strategy in the management of hypertension and other cardiovascular system-related diseases. However, a wide range of their biological effects has turned the scientific interest towards other possible clinical applications of these drugs. The present review demonstrates the available data on the reported angiotensin-converting enzyme inhibitors – based therapies in the treatment of the following human disturbances: cancer, obesity, Barrett syndrome, erythrocytosis, a high-dose-chemotherapy-induced cardiotoxicity, Marfan syndrome, Duchenne muscular dystrophy, migraine, Raynaud's syndrome and Alzheimer disease.

Key words: Renin-angiotensin system, off-label use, cancer.

Introduction

The renin angiotensin system (RAS) serves as one of the most important endogenous regulators of internal homeostasis. Its main functional enzymatic axis comprises renin that processes a liver-produced angiotensinogen to form angiotensin I and angiotensin converting enzyme (ACE) that hydrolyses angiotensin I to angiotensin II in the process that occurs mainly in the pulmonary circulation. Angiotensin II, in turn, being the primary RAS effector, interacts with its specific receptors (AT_1, AT_2, AT_3 and AT_4) to induce pleiotropic biological actions that involve: vasoconstriction of arterioles, hypertension, release of aldosterone, increase of sodium and water reabsorption, release of vasopressin or sympathetic system stimulation. Importantly, these effects are particularly attributed to the systemic RAS that acts within an endocrine axis and responds to general body disturbances associated with water depletion and hypotension. What, however, needs to be emphasized is that apart from the circulatory RAS there are many tissue RAS systems which act in an autocrine/paracrine manner to regulate the structure and function of the corresponding target organs. Thus their actions are distinguished from the ones attributed to the circulatory RAS and they include: stimulation of cell proliferation and growth, inhibition of apoptosis, induction of angiogenesis as well as participation in pro-inflammatory and pro-migratory cellular responses [1, 2].

Given the significance of RAS in the regulation of cardiovascular system function, its blockade by pharmacological intervention occurred as an advantageous clinical approach in the treatment of its various pathologies. Indeed, angiotensin converting enzyme inhibitors (ACE-I), a popular class of antihypertensives, which act by the competitive inhibition of angiotensin converting enzyme leading to the abolishment of angiotensin II production, are commonly used in the treatment of a wide spectrum of cardiovascular and renal diseases, including: hypertension, congestive heart failure, left ventricular dysfunction after myocardial infarction, artherosclerotic vascular disease or insulin-dependent and non-insulin-dependent diabetic nephropathy. Specifically, this family of pharmaceuticals comprises several small-molecule compounds, the most

important of which are: captopril, benazepril, enalapril, lisinopril, perindopril, ramipril, cilazapril, quinapril, imidapril, moexipril and trandolapril. In fact, their clinical efficiency has been confirmed in multiple randomized, controlled clinical trials which showed significant reductions of systolic and diastolic blood pressure in hypertensive patients after their use [3, 4, 5]. However, the family of ACE-I exhibits much more interesting pharmacological profile, associated with their multidirectional mode of action that, apart from the primary repression of angiotensin II signaling, additionally involves potentiation of bradykinin (by the inhibition of its degradation), increase of tissue nitric oxide and prostaglandins, inhibition of matrix metaloproteases, free-radical scavenging and generation of angiostatin. Important to mention that the latter two features are mainly attributed to the ACE-I containing a sulfhydryl moiety [1].

Hence, considering the pleiotropic nature of angiotensin II and the diversity of ACE-I-related actions, there is no surprise that these valuable pharmaceutical agents have been proposed as a potential therapeutic strategy in the management of several other human diseases with the inclusion of: cancer, obesity, Barrett syndrome, erythrocytosis, Marfan syndrome, Duchenne muscular dystrophy, migraine, Raynaud's syndrome or Alzheimer disease. In this review we analyze the available literature data on the off-label ACE-I use.

Cancer

The potential anti-cancer properties of ACE-I stem from their capability of decreasing angiotensin II, the potent stimulus of proliferation and growth. In fact, numerous experimental trials have confirmed the beneficial effect of various ACE-I on the reduction of tumor mass and number of metastases. Specifically, captopril exerted anti-proliferative and pro-apoptotic cell responses in lung and renal tumors [1, 6, 7], enalapril – in human neuroblastoma cells [8] while captopril and trandolapril – in K562 leukemic cell line [9]. Furthermore, the affinity of ACE-I to matrix metaloproteases (MMP), especially MMP-2 and MMP-9 explains their anti-angiogenic and anti-migratory actions. Indeed, these effects have been confirmed for captopril, imidapril and lisinopril in the *in vitro* experiments [10, 11]. In addition to this captopril is known to inhibit tumor angiogenesis by the generation of angiostatin which is a potent, endogenous anti-angiogenic agent [1]. Enalapril, in turn, has been proven to stop cancer progression in the animal model of lung malignancy by the inhibition of TAM progenitors proliferation in the spleen and accumulation in lungs in the mechanism involving the S1P1 receptor signaling [12]. Also several epidemiological data support the introduction of ACE-I into anti-cancer therapy [13, 14, 15]. Nevertheless, due to the lack of appropriately-constructed, randomized clinical trials the utilization of the reviewed drugs in oncology still remains the question of future [1].

Obesity

The impact of ACE-I on the management of obesity is associated with the obesity-related chronic stimulation of systemic RAS as well as with the activation of local RAS system in adipose tissue in obese patients, which contributes to adipocyte growth and differentiation. Additionally, the overexpression of angiotensin II plays a crucial role in the development of the obesity-related hypertension, inflammation, insulin resistance, dyslipidemia and kidney disease collectively defined as a metabolic syndrome. Hence, there is no surprise that the blockade of RAS with ACE-I results in reduced body-weight, improved insulin sensitivity and the decreased serum levels of markers of inflammation. What is more ACE inhibitors have been proven effective in reducing blood pressure in obese humans [16, 17]. Specifically, captopril has been suggested as an interesting therapeutic strategy for obesity, insulin resistance and inflammation [18]. Similarly, enalapril was found to reduce food intake and body fat in young normotensive rats [19].

Bartter's syndrome

Barrtter's syndrome is a rare disease described as an autosomal recesive tubulopathis, involving bilateral hyperplasia of juxtaglomerular apparatus that is responsible for an excessive renin production, hiperaldosteronism, elevated level of angiotensin II and severe hypokalaemia caused by a massive renal potassium loss. In general, the majority of therapeutic approaches in the management of this condition is focused on the normalization of serum potassium concentration by pharmacologic intervention. Interestingly, small doses of captopril have been reported to produce beneficial effects in patients with Barrtter's syndrome with concomitant good tolerance. What is more captopril can be also used in diagnostics of this disease together with scintigraphy [20, 21, 22]. Another ACE-I successfully used for correction of hypokalaemia in this disease was enalapril [23].

Secondary erythrocytosis

Erythrocytosis is defined as the absolute increase in the number of red cells, caused by their overproduction in bone marrow or by tissue hypoxia. In fact, several reports confirm the correlation between RAS activation and increased erythropoiesis associated with secondary erythrocytosis. Hence, the pharmacological blockade of RAS, especially in subjects at risk for secondary erythrocytosis development seems a reasonable therapeutic option. And indeed, as indicated by the clinical data, the implementation of ACE-I-based treatment causes a reduction in hemoglobin levels in a dose-dependent manner in the following groups of patients: individuals with heart failure or chronic obstructive pulmonary disease, patients undergoing hemodialysis, patients after cardiac surgery and kidney transplant [24].

Marfan's syndrome

Marfan's syndrome is a genetic disease affecting connecting tissue that might cause severe complications in heart vessels and aorta, manifested by their stiffness and dilation, which eventually leads to dissection and death [25]. Generally, the advantageous effect of ACE-I in patients with this disorder stems from their impact on the retardation of aortic enlargement progression. Specifically, perindopril has been found to reduce aortic stiffness and decrease its diameters in patients with the reviewed autosomal-dominant connective tissue disorder [26].

Duchenne muscular dystrophy

Duchenne muscular dystrophy is a genetic disorder caused by the lack of protein, dystrophin, which leads to muscle degeneration. A gradual decrease of myocardial function is one of the consequences of this condition [27]. Interestingly, ACE-I have been found to delay the progression of cardiomiopathy in dystrophic patients while the combination of ACE-I and beta blockers has provided a benefit of a long-term survival within this population of patients treated against heart failure [28, 29, 30].

Prevention of a high-dose-chemotherapy-induced cardiotoxicity

As is well known, the intensive anticancer treatment is inherently accompanied by multiple serious side effects which compromise the effectiveness of this medical procedure. Specifically, chronic cardiotoxicity is the major reason for the limitation of athracyclines-based chemotherapy, which further affects patient survival. As a matter of the fact, an important role in the development of anthracycline-dependent cardiomiopathy is actually attributed to cardiac RAS, which indicates that the introduction of ACE-I into treatment could provide an effective prevention against chemotherapy-induced cardiac dysfunction. Indeed, in one clinical study the administration of enalapril to the patients at high risk of cardiac insufficiency receiving high-dose chemotherapy resulted in a reduced number of cardiac-related adverse events, prolonged patient survival and stabilization of cardiac function. The mechanism responsible for this effect could involve the enalapril-dependent decrease of systolic wall stress, reduction of afterload, atteuation of fibrosis, inhibition of cell apoptosis and free radical scavenging [31, 32, 33, 34].

Migraine

The relationship between migraine and RAS is generally not fully recognized yet some interesting observations indicate that the frequency of attacks without aura positively correlates with the activity of ACE. Also there are several literature reports showing that the administration of lisinopril substantially improves the incidence of headaches in hypertensive and normotensive patients suffering from this condition. Similarly, enalapril has been found to exert a clinically significant prophylactic effect in patients with migraine headaches. Unfortunately, the putative mechanism of action behind this pharmacological response remains unknown yet certain data indicate that it could be the result of the ACE-I-dependent altered sympathetic activity, decreased degradation of bradykinin, encephalin, and substance P, increased prostacyclin synthesis, and free radical scavenging [35, 36].

Raynaud's syndrome

The Raynaud's phenomenon is characterized by a reversible peripheral vasospasm in response to cold, which usually affects hands and feet. The pharmacotherapy of this clinical condition focuses on vasodilatation and in this case ACE-I seem to offer a promising mode of action. Unfortunately, the available clinical trials provide conflicting results with several reports that emphasize the improved digital blood circulation after long-term and acute treatment with captopril [37] and other ones that demonstrate no influence of

captopril or enalapril on the severity or frequency of disease attacks [38,39]. Hence, it is currently believed that although ACE-I might provide some minor effect to alleviate the disease symptoms, they are not superior to the traditional treatments. Thus, the appropriate clinical trials are needed to conclusively establish the role of ACE-I in the management of Raynaud's phenomenon [40].

Alzheimer disease

The potential contribution of RAS to the development of Alzheimer disease stems from the local expression of this system in brain. In fact, there are several experimental data suggesting the participation of ACE in the metabolism of beta-amyloid protein, the reduction of which occurs in this pathology. Interestingly, the administration of ACE-I has been found beneficial in the prevention of dementia or cognitive decline in humans without any symptoms of dementia while in patients with amnestic mild cognitive impairment, ACE-I produced stabilization of cognitive function. Furthermore the administration of ACE-I to adults suffering from Alzheimer disease has been found to delay the rate of cognitive decline. Contrary to this there are other reports suggesting the ineffectiveness of ACE – I in the treatment of this clinical condition, which indicates that the utility of these drugs in patients with Alzheimer disease remains questionable [41].

Conclusion

The diversity of biological actions determines the variety of possible ACE-I's clinical applications which reach far beyond the classically-accepted use in hypertension and the related cardiovascular diseases. The primary limitation, however, in the extension of their clinical indications is lack of appropriate, randomized, controlled, long-term clinical trials that could definitively confirm their utility in the management of the above reviewed diseases.

Conflict of interest

The authors declare no conflict of interest.

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REVIEW PAPER

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The history of the Human Immunodeficiency Virus research

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ABSTRACT

The authors summarize the current knowledge of the beginnings of the human immunodeficiency virus (HIV) infections. Most of the studies have so far supported the theory that HIV infections had in their initial years been a typical zoonosis which had been present among African tribes for over 300 years. Most likely, infection was transferred from monkeys, particularly from chimpanzees, on multiple occasions. The most recent publications allow us to describe the transfer of the virus into humans, and new epidemiological data allow us to carry out analysis of the global spread of the virus. Studies of histopathological samples taken from patients in the 1960s have cast new light on the issue of virus presence in the US population, and the previous theories tracking the beginning of infections to the 1980s have had to be modified. Greater awareness of pandemic mechanisms should allow for more effective future counteraction of the spread of new pathogens.

Key words: AIDS, HIV, SIV, zoonosis.

Virus isolation

In 2013 30 years has passed since the identification of the HIV virus as a cause of immune deficiency. The rapid increase in the incidence of diseases associated with immune deficiency in the early 1980s prompted molecular biologists and virologists to carry out coordinated research on a previously unknown scale [1].

In 1983 Luc Montagnier, head of the virology laboratory at the Pasteur Institute, with his research team, including Francoise Barre-Sinoussi, isolated a virus from the blood of infected subjects and called it lymphadenopathy associated virus (LAV). The virus, isolated from the lymph nodes of subjects at an early stage of lymphadenopathy, was found to have reverse transcriptase activity, and was classified as a retrovirus. *In vitro* studies demonstrated that LAV had the potential to infect lymphocytes of healthy individuals, and reacted with antibodies isolated from patients with advanced immune deficiency syndromes, which finally confirmed its pathogenicity [1].

One year later, an independent study by Robert Gallo and his team carried out in the USA led to the isolation of a virus named human T lymphotropic virus type III (HTL-III) [2]. Another year later, in 1985, researchers found that both identified viruses were the same, and in 1986 they were given the name of the human immunodeficiency virus (HIV-1) [3, 4]. The world of science recognized the discovery of the HIV virus by awarding the French researchers the Nobel Prize in medicine in 2008 [5]. Despite the scientific consensus, there were some separate voices, that argue correlation between trends in drug abuse and AIDS cases [6]. Prof. Peter H. Duesberg states the theory that HIV is a harmless bystander of AIDS [6]. That theory has been rejected by the scientific community.

Further development of large-scale studies resulted from the spreading epidemics of various diseases which had previously been diagnosed only in people with immune deficiency, mainly during immunosuppressive treatment. The first historical records on atypical infections are found in the epidemiological reports from the Centers of Disease Control (CDC) concerning pneumonia of a rare aetiology (*Pneumocystis jirovecii)* diagnosed in five homosexual men in Los Angeles, and

in patients with haemophilia A who had received clotting concentrations and who developed pneumonia of mycotic aetiology, not previously recorded [7, 8].

In 1987, genome of the virus responsible for the acquired human immunodeficiency syndrome was isolated from the West African population and sequenced [9]. Despite the similarity between the clinical manifestation of AIDS in the African population and that among American homosexuals, the weak cross reaction of antibodies only pointed out the distant relationship between the two strains [9]. According to the established nomenclature the microorganism was given the name of human immunodeficiency virus type 2 (HIV-2) [9].

HIV infection as a zoonosis

The search for an animal model of AIDS led to studies on the virus previously known as simian T-cell lymphotropic virus type III (STLV-III) [10]. Researchers found a close genetic relationship between STLV-III and HIV-2, and named it, according to the existing nomenclature, the simian immunodeficiency virus (SIV) [10]. As standard, a suffix denoting the species from which the virus was isolated is added to the virus' name, e.g. SIVmnd for mandrill, SIVsmm for sooty mangabey, and SIVmon for mona monkey. The vast majority of SIV infections in monkeys were asymptomatic. A significant discovery was the identification of the SIVcpz virus originating from the chimpanzee [11]. Unlike the initially isolated SIV, the SIVcpz strain demonstrated an antigenic similarity with HIV-1 [11]. The phylogenetic analysis demonstrated that strains originating from chimpanzees and macaque monkeys are closely related to HIV-1 and HIV-2 viruses, respectively [11, 12]. The role of these viruses in transmitting infection to humans was confirmed by *in vitro* studies which supported their potential for infecting humans [13]. Research supported the theory that HIV-1 and HIV-2 infections had in their initial years been zoonoses which had occurred for thousands of years among West African tribes hunting for primates [14].

Spread of the epidemic

Endemic infection with HIV-1 and HIV-2, previously limited to the population of central and western Africa, spread to the entire sub-Saharan region due to colonial expansion by European countries and resulting population changes [15]. Also, the accelerated spread of epidemics occurred after the introduction of parenteral antibiotics and vaccines delivered in unsterile injections [16]. In the early 21st century a popular theory coined by Hilary Koprowski attributed the epidemics of the HIV-2 infection to the widespread use of the polio vaccine. Concerns were raised with respect to the cultures of cells derived from kidneys allegedly originating from infected chimpanzees. However, the theory was strongly rejected after the complete analysis of the vaccine's manufacturing process [17]. The epidemic's first outburst occurred in Léopoldville (now Kinshasa) in Congo, and David Carr, an English printer working in Africa in the 1950s, who died in 1959, was the first patient diagnosed with AIDS based on tissue samples [18]. Sharp et al. analysed the risk of the spread of HIV outside the African continent, and pointed out the fact that the spread must have occurred before 1940 [15]. Haiti was the first place in the Western hemisphere with a confirmed incidence of AIDS. The analysis of pathomorphological specimens originating from deceased patients allowed for estimation of the arrival of HIV in Haiti as about 1966 [19]. Through intensive commercial contacts with the Republic of Congo the population of infected people in Haiti increased gradually, while economic migration to the USA caused the spread of the virus among the American population three years later [19]. In the USA, Robert Rayford, a 16-year-old Afro-American from St. Louis, Missouri, was the first confirmed fatal case of HIV-1/AIDS [20]. Rayford died in 1969 due to opportunistic infections, and also suffered from Kaposi's sarcoma. Doctors treating Rayford during his hospitalization found that he was homosexual, probably a male prostitute, and had engaged in repeated receptive anal intercourse [20]. The risk of infection due to blood transfusion was excluded, thus suggesting that the HIV-1 virus has been present in the homosexual environment since the early 1960s [20]. In the same year in Europe, a man whose true identity is officially unknown died, and tests carried out 20 years later confirmed that he was infected with the HIV virus [21]. It is known that the man was a Norwegian sailor who had visited Africa on many occasions, and had been treated earlier for gonorrhoea. Tests carried out later confirmed infection with HIV-1 in his daughter and wife [21]. It was established that after ending his career as a sailor the patient worked as a lorry driver throughout Europe, and was sexually active with prostitutes, mainly in Germany and Denmark [21]. Gaëtan Dugas, a Canadian airline steward, was referred to as 'Patient Zero' in the first epidemiological AIDS study [22]. Auerbach et al., who analysed the spread of HIV epidemics, concluded that the virus must have been transmitted by a person engaged in numerous homosexual contacts throughout America [22]. The analysis drew attention to the fact that Gaëtan Dugas had homosexual partners in California, New York, and several other states

in America. Dugas died in Quebec in 1984 as a result of kidney failure caused by AIDS infections [22]. The initial theory implying that Dugas was the first person to bring HIV to the American population was not confirmed. Also, his role in bringing HIV to Los Angeles and New York is currently being questioned due to the short time between sexual contact and the onset of symptoms in his partners (10.2 months), because later studies estimated the period to be on average 10 years long [22]. Dugas claimed to have had at least 2,500 partners across North America in the 12 years of his sexual activity, which shows the scale of sexual partner exchange in homosexual environments in the 1970s [22].

Summary

The history of research on HIV epidemics shows how the globalisation of lifestyle in the 20th century influenced the spread of endemic infections. Studies carried out to date demonstrated that for at least 8,000 years SIVckp and SIVmok viruses, occupying the same ecological niche, have been transferred from species to species and have infected Homo sapiens. In addition, high genetic variability allowed viruses to develop the potential for vertical and horizontal infections, which for a long time were restricted to tribes endemic in today's Cameroon and the Republic of Congo. Colonial movement in the 20th century led to the establishment of large urban centres connected via marine routes with countries in Europe and America. Kinshasa, being a port city, was the first confirmed location from which epidemics spread [15]. The efficient work of the Centers for Disease Control (CDC) led to the identification of the disease and within a week they released a report on atypical cases of pneumonia among homosexuals and persons receiving blood substitutes, prompting the scientific environment to search for pathogens [7, 8].

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REVIEW PAPER

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Analysis of benefits from being in a Alzheimer Internet support group for the caregivers of the people suffering from Alzheimer's disease

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ABSTRACT

Both emotional and physical demands faced by Alzheimer's disease caregivers are very difficult to meet. Often, the amount of strength and calmness needed to fulfil the role of caregiver is beyond the person's adaptability. The aim of this article is the data analysis of the Alzheimer's disease caregivers online support groups and problems the caregivers face on the daily basis. Authors compared discussions and solutions available in polish language with methods used in other countries. The study was conducted in late April and May of 2013 by analyzing the user posts found on Polish webistes that associates the caregivers of people suffering form Alzheimer's disease. Authors assumed that there is a difference between citizens of different countries in the access to useful information that may help in solving daily problems. Confirmation of this hypothesis may indicate the need for modifications by creating a professional platform that associates Alzheimer's disease caregivers. Authors also analyzed remedies caregivers use and emotional functioning. By presenting recurring problems regarding diagnosis, burnout and coping with daily stress. They try to answer important question: what must be done to meet the needs of caregivers presented via the message boards. They are trying to prove that access to professional knowledge and presence in the environment that brings together caregivers can significantly improve level of performance and satisfaction. Even if the aid is granted only through an online platform of information sharing, the support effects are clearly visible.

Key words: Alzheimer's disease, caregivers, discussion boards, support groups.

Introduction

The aim of the paper is the presentation and review of the Alzheimer's disease caregivers problems. The requirements facing the caregivers of the people suffering from Alzheimer's disease are very challenging and cost a lot of effort. Senile dementia is an illness that hurts both the patient and his/her family. Taking care of a person with an Alzheimer disease requires a great deal of sacrifice – the person needs to be washed, dress up, fed etc. It has its social implications as well – one has to give up the social life he/she had and change family relations. As the result the caregiver often becomes alienated and left alone. The nurturance also leads to greater amounts of stress – the caregiver might worry what happens if he/she gets sick. Often the family members worry also about their kids being around a person suffering from Alzheimer's disease. The sick person requires all-day-long care, forcing the caregiver to sacrifice their work for the wellbeing of the sick person. Often they do not have sufficient funds to guarantee a necessary comfortableness to the person with dementia. Additional protections around the house, rails to help one move around, medicine drugs – it all requires enormous financial investment.

In Poland the frequency of Alzheimer's disease distribution increases by 1% among people between 60–65 years old, and even by 40% among people aged 85. Currently around 400 thousand people are suffering from the dementia, among which 50–70% deal with Alzheimer's disease [1]. The number of people suffering from dementia is steadily rising while the number of proper health centres stays the same. Only about 3% of patients with dementia live in educational care centres and only 6% of the caregivers are satisfied with those organisations [2]. 92% of the patients stay at home, being taken care of by their family for whom it becomes a great burden, as already explained. If often happens that the caregivers are partners of the sick person, who also reach the age of 60 and are not really able to properly take care of their loved ones. As a result, dementia has two faces-both in the form of a sickness hurting the patient and negative effects and burdens hurting the caregivers [3].

All the mentioned facts and the problems outlined resulted in caregivers' communal decision to associate and create their own space, the most accessible one in the XXI century – on the Internet. It is precisely the Internet, namely all the forums, blogs, virtual support groups and online editorials, that became the place where caregivers try to help one another. Believing that only a person in a similar situation is able to understand their suffering, they try as much as they can to exchange knowledge, advice, experiences, share their emotions, challenges and search for contact with another human being. People are being shuffled around from one doctor to another until they find the forum where they can find the answers they were looking for- more or less satisfying. Net surfers ask about symptoms, how to understand them and how to behave around Alzheimer's patient. They seek general information and ask about individual cases. They post that they lack someone who could monitor the patient and help with the duties around the patient. They complain about losing their own life while searching for friends online. On top of that, aside of standard queries, sometimes they ask questions that are much more individual in nature, which touch on problems, occurring less often.

Due to high ratio of people suffering from dementia to professional caregivers and because of the lack of sufficient financial support from the government it is virtually impossible to solve every problem that the caregivers might face. However, it is crucial to at least deal with the basic challenges, such as professional diagnosis and guidelines, whilst in terms of other challenges (psychological, physical, financial) none of the caregivers were left alone. It is worth underlining that professional advice and kind words are extremely valuable and helpful-whether they are spoken out loud or written on the Internet forum. The issue raised in this paper is about facing the challenges and needs described by the Internet users.

The picture of the environment of the caregivers of the people suffering from Alzheimer's disease

XXth century has brought dynamic development of the idea of mutual-aid and creation of numerous movements and organisations dealing with this issue, hence various systems of self-support for people with similar problems, including health problems. Those ideas sprung from the imperfect and insufficient health-care systems, not dealing with needs of the sick people, and lack of social support in the traditionally practiced medicine [4].

Among the people taking care of the Alzheimer's patients exist many movements or forms of self-help. There are support groups at the hospital wards, rest homes, hospices, institutions of social help etc. On the Internet one can find multiple sites for the caregivers of the dementia patients, on which we often find fragmentary knowledge, hardly useful tips etc. That is yet another reason explaining why caregivers associate themselves using the Internet forums.

Within the Internet the caregivers most commonly ask questions about a very specific symptoms of individual patients. Often it is possible to find the whole history and progress of illness for particular people. Sharing all the information about the sickness might therefore serve as a form of education. Caregivers experience complete lack of or insufficient amount of real help through sharing information, organising various forms of rehabilitation, cooperation with families, professionals, institutions, and sometimes financial help. The Internet forums also feature pieces about physical and psychological exhaustion of the caregivers themselves.

Taking care of the family member with dementia is very burdensome; the caregivers must show great patience and understanding. They are usually solitary in their struggle, forced to the continuous, all-day-long nurture over the sick person. As a result they are practically isolated from any social life. The behaviour of the sick person is highly unpredictable; hence the caregivers must continually face new challenges and situ-

ations. To deal with them he/she needs professional support and knowledge. Just like Mrs Jolanta explains: "the sick are lost in their surroundings, they act abnormally and irrationally, weird even, i.a. they hide different objects in the strangest places, and then they try to find it [...] The sick keep repeating something stubbornly, they are hyperactive, even aggressive sometimes or they decline carrying out the most basic activities, like washing up [...] Such behaviour shocks and surprises, for the people around they seem funny or are suspected of acting out of spite, they cause dejection for the caregivers, and nervousness – says Mrs Węsierska [...] Often, though inaccurately, the Alzheimer's disease is associated with psychological illness, while it is a neurological disturbance [...] What is needed are drugs, therapy and lots of patience" [5].

Such intensive and burdensome care must put destructive pressure on the family life, career and social relations of the caregiver, resulting in many offshoots of the drawn-out stress, like: insomnia, irritation, anger, shame, loss of feeling of self-worth, guilt, depression, and also aggressive (both active and passive) behaviour towards the sick. It also results in frequent occurrence of serious health conditions among the caregivers associated with heart problems, arterial hypertension, joints' degeneration, diabetes, or gastric disturbances. "It has been proved that taking care of the dementia patient is more burdensome and stress causing than taking care of the people with somatic illnesses" [6]. This burden becomes even greater in the mentioned situation of financial problems, lack of sufficient knowledge about the sickness and the rules of care, inability to cope with new and escalating health problems of the sick person.

The caregivers often find themselves misunderstood by the family members or other people, which might lead to isolation in action. Internet forums point out to numerous problems with which the caregivers of the Alzheimer's patients must face and how important for them is their mutual psychological support and how they need all the forms of real help. "Hence the inseparable element of dealing with the dementia patient must be a psychological and material support of his/her caregiver, and ensuring the support of other people and regular rest [...] Support groups, which should be attended by both the caregivers and the sick, allow for emotional abreaction from the care and exchange of experiences, and also supply practical advices about the progress of the sickness [...] The caregiver can also use the legal advice, information services about the nursing methods, and rehabilitation equipment" [6].

Analysis of Internet forums content – caregivers' problems

The issue is extremely problematic from the very beginning. First of all, people see the change in the behaviour of a close person but are unable to diagnose it properly. They are not certain whether it is the right moment to contact the specialist. This issue is especially problematic when dealing with the older people. Many assume that old age has its laws and hence cannot distinguish between dementia and the negative influence of aging.

Another problem presents itself when the caregiver realizes that the sickness has probably started and tries to consult with the doctor. The wards often treat visits to the hospital with great suspicion and it often happens that they decline to consult the doctor. Even if convinced otherwise, they tend to assert that everything is just all right.

How to take care of such a person? Many caregivers of the dementia or Alzheimer's patients frequently ask questions on the Internet forums dealing with this problem. They rather bring up issues of a specific behaviour. Hospitals often have their own, specialised ways of dealing with such situations, yet no one shares this information or gives advice to the caregivers how to deal with them in the home environment. Aside of issues like feeding the sick that does not want to eat, or changing the diapers, what caregiver often ask about are: controlling the finances of the sick, giving medicine, general advice behaviour, where to look for support, information and advice, the lack of reliable information about the sickness and possibility of support.

Demanding and long-lasting care over the sick is extremely engaging and exhausting. It creates a myriad of problems that are emotional and even psychological in nature, e.g. stress, inability to cope with new situations, inability to keep one's distance, helplessness and problems with physical health.

The caregivers often deal with numerous angsts and moral dilemmas. Many Internet posts show great doubt towards the nursing homes. Aside of financial issues, people are afraid of bad treatment in such facilities. Many also face the feeling of guilt believing that putting a relative in a nursing home is simply getting rid of him, often not realising that care at such facilities usually is much more *stress less* and safe than at home.

The frequent consequence resulting from taking care of the sick is resigning one's work. Significant number of people writing on the Internet forums informs that they needed to give up their careers for

the sake of taking care of the older person. Often it is a result of indifference of the rest of the family, which forces one person to take the entire burden. The caregivers then sacrifice themselves entirely in the feeling of duty. As the consequence of spending the whole day with the ward is losing friends, which in turn results in feelings of loneliness and depression. Losing the social skills by the people who take care of the sick person for so many years change their functioning style so much that they are unable to function in normal conditions.

The reaction of the environment turns out to be no less problematic than that. It is often very negative in nature. People often do not know what the disease leads to and how it operates which results in inadequate judgments of the Alzheimer's patients, and other dementia-related diseases, and strong criticism of the family. Some also do not appreciate the burden that the caregivers need to deal with- they accuse the family of not paying a proper attention, neglecting the sick, of deliberately mistreating the patient.

Often a long-term, all-day-long care of the sick person leaves a mark on a caregiver's psychic. Such people often complain about various psychological disorders. The tragedy of the caregivers usually does not even stop when they cease to take care of the sick. It continues even when the patient is sent to the nursing home (Table 1).

Possible solution of the problem

Based on the analysis of the situation in the Anglo-Saxon countries – The Great Britain – one can see that there are numerous Internet sites dedicated to the Alzheimer's disease which also include expanded sections about support for the caregivers. In this chapter we will cover ways of providing Internet support functioning in other countries. We will also evaluate the need for providing the Polish equivalent of those sites, offering support to the people engaged in taking care of the sick.

The Internet provides extended guidebooks informing how to treat the sick person, how to take care of one's own physical and psychological health, and also how to plan financial expenditure. Those guidebooks are available both in the form of plain text and multimedia [12, 13, 14].

At the Alzheimer's Association website one can find questionnaires enabling the initial diagnosis of the emotional state of the caregiver and a telephone support line for the caregivers. The people handling the support line offer wide-range of support from offering the basic information regarding i.a. cognitive deficits occurring at the initial stage of Alzheimer and explain how to deal with crisis situations [12].

The foreign websites also enable access to the database of specialist agencies and institutions that deal both with personal problems of the caregivers and that of the patient that exceed the abilities of the caregiver, but requiring a professional health service [15, 16]. There are also Internet applications available, among which are [12]:

- Those that enable planning the future events for the caregiver and the Alzheimer patient – the programme aims at inspiring awareness in terms of future needs such as: adjusting the house to patient's needs or car driving
- Event-planner/Calendar
- Virtual library – containing the collection of scientific research and articles dealing with Alzheimer disease.

American group called "Alzheimer's Association" on its website offers access to a wide range of multimedia resources, including educational videos. It is possible to find statements there of distinguished academics on the important role of early diagnosis in Alzheimer patients, current research [12], or effectiveness of various medicines [12]. On top of that, people seeking support may access videos with useful advice on an appropriate diet, rich in antioxidants, or the most advantageous physical exercise for the sick patient [12].

The caregivers of the Alzheimer patients, by signing up on the website [12], give their consent to receiving a weekly newsletter created by the association's members. It is an email, which contains the most recent scientific and political news, so information about newest scientific discoveries and government activity for patients with dementia. Additionally, the e-newsletter contains information about a newly created support groups, trainings for the caregivers or short workshops such as "basic communication skills with Alzheimer patient".

Majority of the foreign websites dedicated to Alzheimer's disease allow virtual contact with specialists in this disease. And so, for instance, the American foundation for Alzheimer patients offers a service called: "Ask the expert" [18] on its website. The specialists offer professional advice on-line and answer questions of the people engaged in taking care of the sick, treating each case with an individual approach. The website also features the ranking of the most often discussed issues and professional advice of very qualified people. Visiting the website "Alzheimer's project" [19]

Table 1. Problems and caregiver messages. Source: Own

Negative influence of aging	"After the death of my grandfather, grandmother got her first stroke, yet she recovered with only slight brain damage and we did not detect any changes- trivially we missed the beginning of a sickness" [7].
Consulting the doctor	"Father does not even want to hear about seeing the doctor [...] after seeing the neurologist the doctor wanted to keep him for a 3-day observation and do a X-Ray computed tomography with contrast agents but he signed his release for his own responsibility and the neurologist could not do anything about it. Anyway, it does not really matter because once father visits a doctor he starts lying and distorting the facts, claiming that everything is alright, that only his knee hurts" [8].
Controlling the finances of the sick	"We are afraid of doling out money, or that someone will skew the grandmother when they see her problems when dealing with cash. Would it be a good move to pay the money into a bank account for safety?" [7]
Giving medicine to the sick person	"I took my mother's medicine and hid it well, she won't be pilfering it anymore (...) only now she keeps arguing about those drugs" [9]
General advice behaviour	"I am looking for any advice regarding how to hush her. Can our behaviour have any tangible impact on her?!" [9]
Lacko of information where to look for a support	" Many posts on the Internet forums are titled "lack of professional help". One of the women wrote: "we travel to the doctor with the mother in law but we lack support to know how to deal with such a person even at home, so that there would not be any life-threatening situations" [10]
The lack of reliable information about the sickness and possibility of support	"Maybe I am expecting too much but so far none of the doctors could tell me anything about the illness that I did not already know myself" [9]
Psychological problems such as stress	"I live in constant stress. Anxiety. I constantly feel guilty. I cannot deal with it anymore. I am heartbroken...I suspect that stress is what caused my two previous miscarriages"[9]
Inability to cope with new situations	"We were so close together once and I do not deny that I am dealing with it terribly...I see that I now exist only in his memories" [9]
Inability to keep one's distance	"I see that mother cannot cope with it. Father's every action and behaviour discomposes her, she cannot distance herself." [9]
Helplesness	"The grandmother does not listen to anyone. Even the grandfather has lost any influence over her. We feel helpless" [7]
Problems with physical health	"We are psychologically exhausted, but we are starting to lose our physical health as well. We need to carry the grandfather to the toilet" [9]
Fear of wrong diagnosis	"I am terrified with the fact that doctors state their diagnosis so easily because it makes it so much harder for us to take care of him" [9]
Losing friends	"Lack of contact with other people sometimes drives me crazy"; "I could forget about having friends, only a handful remained. My love will not last, but I am not surprised." [7]
Losing social skills	"I am terrified that I can no longer live normally, and now it is a pure prison for I even do not leave the house [...] I have no more patience for the healthy, living people, I avoid them as much as I can." [11]
Family neglection	"Usually about twice a week the neighbours (suddenly eager to bring help) call the police because we are "murdering the old lady". We heard: "God has punished you!", "You do not show enough understanding!", "There is a mad person in the house!", "It is sickness! What will you do when you become sick?!", "It is your duty!", "You should be doing what she wants, you owe her that!" [10]
Long-term, all-day-long care of the sick person leaves a mark on a caregiver's psychic	"Now A. is at the nursing home, we did not deal with it. I thought that everything will change now, but in the meantime...everything became so irrelevant, I became indifferent to everything. I really cannot pull it together." [7] "Mother's son came back to work. And what about me? I stayed all alone in the empty house. Then the all-encompassing silence made me realise that the challenge has been finally completed. At the finish was only a deep wound in the heart, the burden of painful experiences which you cannot forget...and all-encompassing abyss" [11]

we can find another possibility of having an on-line chat with one of the experts. The mentioned options make the flow of information between the people who need it – the caregivers – and those who offer it – specialists- very fast and fluent.

Volunteering is another interesting and useful option. In 1991 reverend John Fletcher, the Methodist pastor from Florida, introduced an innovative support programme which today is known as: "Alzheimer Project". Initially, the range of the services provided was very narrow and included mainly support for the caregivers within the parish. Currently, Alzheimer Projector operates in 12 counties in Florida, uniting people who want to help others. The idea of the organisation focuses on finding volunteers who can fill in for the caregiver and take care of the Alzheimer patient. People who want to perform such activity must register at the volunteer's base/headquarters and undertake a training preparing them for their future role. Then the volunteer is sent to the family that approached the organisation asking for help in order to spend few hours with the sick and substitute for the caregiver. Thanks to such services, the caregivers finally have time for themselves. The manager at the Alzheimer's Project underlines how important for the caregivers is the ability to take care of their own psychological and physical well-being. The volunteer visits a household once a week, taking care of the sick for 2–4 hours. The caregivers may spend this whole time on whatever pleasures he wishes, but for which he did not have time beforehand: from a long, afternoon to engaging in a long-forgotten hobby. The participants of the Alzheimer's Project call such visits the "rest time" [20].

Based on the foreign experience from Canada, USA or the UK we propose construction of the national web portal of the same character. When analysing the Polish interest resources dedicated to dementia diseases one realises the lack of sufficiently developed website offering the necessary information about the Alzheimer disease and advice for the caregivers. It does not mean that such information does not appear on the Internet- for instance there is an Internet guidebook that can be found on the alzheimer-poznan.pl website. [21] It is necessary to create an extended Internet platform dealing with Alzheimer's disease and offering support for the caregivers in Poland. Its core should be a fully featured guidebook containing information about:

- taking proper care of the Alzheimer patients
- taking proper care of one's own physical and psychological health when taking care of the Alzheimer patient

The information should be presented both in the form of text and multimedia. Among those materials there should be companions dealing with:

- taking care of physical and psychological health of both the caregiver and the patient (the proper diet, advantageous physical exercises, how to lower the stress level, how to deal with the feeling of loneliness)
- financial planning
- proper treatment of the sick person (time-planning based on patient's abilities, allowing for the greatest elasticity of his/her movements, organising patient's time and activities)
- problems and deficits that may occur (like memory loss).

The crucial issue is including the information about the medical institutions helping people with Alzheimer's disease. The caregivers could save a lot of time that would be otherwise wasted on trying to find the specialist help.

The website should also offer a possibility of contacting the psychologist. The support could be offered in either the form of e-mail (when dealing with minor issues) or Skype conversation (when dealing with serious situations and mental conditions of either the caregivers or the patient). The psychological support may also be offered indirectly through database of the therapeutic support groups.

The stress reduction and challenging the feeling of loneliness, besides the help of psychologist, may be offered by an Internet forum for the caregivers. Contacting other caregivers of the Alzheimer's patients would allow:

- exchange of precious information and advice
- acquiring support from other caregivers
- improvement of the psychological state through altruistic help offered to others
- discovering that caregivers are not solitary in their suffering.

How to utilise the idea of the pastor from the Methodist Church in Florida and implement it in Polish reality? Besides educational and informational services, the website should associate people who would like to help. The volunteers from different regions of the country through online registration would be added to the Internet database of the people offering their help and time for the families of the Alzheimer patients and patients themselves. The Internet search engines would allow the caregivers to find a volunteer in their closest neighbourhood. The project could also benefit greatly if the people from parish associations and student societies could also be engaged in the project.

Conclusions

In the light of presented information we can see that taking care of Alzheimer patients/people suffering from Alzheimer requires enormous physical and emotional sacrifice. The requirements for the caregiver change on daily basis. He/She must dramatically change one's social life, i.e. relinquish the social relations and adjust one's free time to the needs of the sick. The caregiver may apply a long-term strategy based on acquiring a sufficient knowledge that would help in a day-to-day nurture of the sick. Training programmes teaching families about the development of the Alzheimer's disease and practical strategies dealing with crisis situations would be of great help.

Adjustment to new situations, skills learned through experience and strong web of support from the family may in substantial way help the caregiver in their daily struggles and care of the loved ones. Support groups also constitute an important element in the functioning of the caregivers. They allow the members to take a rest from their duties and burdens, and to share their doubts, exchange experiences, acquire an advice and get emotional support and understanding. The webs of self-support may be especially helpful when the caregivers face extremely difficult decisions and have nobody to talk to. Diagnosing the Alzheimer's disease leaves people with myriad of questions, to which answers are neither easy nor desirable. Getting solid information is impossible or they are not sufficient enough in a current situation. It is not just the patient's health that is problematic, but so are the finances, security, legal issues and planning the future. The caregivers must also remember about their own health and well-being. A proper diet, physical exercises, and stress-relief activities are important part of it.

At the time of a rapid technological development of the Internet there is a need emerging to create a web-based platform that would serve as the web of support associating all the caregivers dealing with people suffering from Alzheimer's disease. Thanks to analysis and evaluation of the statements of the caregivers made on the Internet we see a great need for creating a homogenous Internet service that will allow to acquire a sufficient knowledge from the professionals. However, above all such portal should facilitate the creation of associations of caregivers and the sick. It should be a community shaped on the basis of solid knowledge that the members could trust without a doubt. It will supply a necessary level of support and help necessary when working with the sick. What is more, it should be a platform that the caregiverscould access to find the newest knowledge and discoveries about those illnesses, including their progress and ways of restraining their development. It should unite the volunteers and allow them access to information about the people seeking their help. In the future it should coordinate actions of the volunteers with people seeking help- so that is becomes a place where caregivers could declare the need for volunteer help in real world. Finally, it should supply information about local facilities and institutions for both the caregivers and the sick. We hope that in Poland, as we have seen in the proud examples in the West, we will also have a professional platform of support for the caregivers that would meet their needs and requirements.

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REVIEW PAPER

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Expressions of genes encoding steroidogenic enzymes and their role in prostate carcinogenesis

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ABSTRACT

The concentration of sex steroid hormones in the prostate gland is controlled by their local synthesis and metabolism. These processes involve steroid metabolizing (steroidogenic) enzymes, which are necessary to produce the active form of androgens and estrogens at specific locations. Changes in gene expression of the steroid metabolizing enzymes may play an important role in prostate carcinogenesis by regulating sex steroid concentration in the prostate gland. The purpose of this review is to gather the most important reports connected with gene expression of the steroidogenic enzymes and to find correlations between gene expression and tumorigenesis in the prostate gland.

Key words: prostate cancer, steroidogenesis, steroidogenic enzymes, steroid hormone.

Introduction

Malignant neoplasms, as the second most popular cause of death worldwide after cardiovascular diseases, pose a serious health problem. One of the most common malignant neoplasms in men is the prostate cancer *(PCa)*. In 2009 in the United States 192,280 people were diagnosed with this cancer, of whom 27,360 died [1]. In 2006 in Poland over five thousand patients died of PCa, out of the total 71,000 patients diagnosed with neoplastic diseases. Thus, PCa was the third type of cancer with the highest mortality rate [2]. Despite high prevalence of PCa and numerous scientific research programs, the mechanisms underlying its development still remain unclear. The most important risk factors of the prostate gland malignancy include age, race and family history of the disease. PCa is rarely found in people below forty, but above that age the incidence rate increases dramatically, as compared with any other type of cancer. Apart from age, an important factor predisposing to PCa is the race. The most vulnerable ethnic group are the African Americans, and the least the Asian population, which may be associated with their diet and lifestyle. The risk is also increased in the cases of family history of PCa, which is probably related to genetic factors [3]. Steroid hormones may also be important in the PCa development, as their presence is essential for a proper functioning and growth of the prostate gland. The steroid hormones are supplied to the prostate with the blood flow, but thanks to the local expression of steroidogenic enzymes the steroid hormones can be also synthesized and metabolized within the gland. Variations in the expression pattern of the steroidogenic hormones during neoplastic transformation may significantly affect the intracellular concentration of steroid hormones in the prostate gland and thus play a role in the cancer pathophysiology [4]. Identification of possible gene expression alterations within the prostate, crucial for extragonadal synthesis of steroid hormones, may be helpful in better understanding of PCa development mechanisms. Consequently, this may be a starting point for working out new therapeutic approaches, based on analogs or steroidogenic enzyme inhibitors.

Steroid hormones and the prostate cancer

The role of steroid hormones in the prostate gland carcinogenesis became the focus of scientific research in

1941, when Huggins and Hodges demonstrated the effect of surgical castration and anti-androgen supply on a reduced level of acid phosphatase in the blood serum of PCa patients. Acid phosphatase was the first biomarker, whose elevated level indicated the PCa. Additionally, higher concentration of acid phosphatase was noticed after androgen injection in patients with advanced PCa [9]. These studies identified testosterone as a major factor involved in the neoplastic transformation of the prostate cells, and initiated the use of a therapy aimed at inhibition of testosterone biosynthesis. In most cases, blocking testosterone production by means of surgical or pharmacological castration results in initial tumor regression, but over time the tumor becomes androgen-independent [10]. Subsequent studies have provided further evidence of the androgen role in PCa pathogenesis. It was demonstrated that the incidence of PCa in patients castrated before puberty were extremely rare [11]. Moreover, the effect of DHT on the regulation of proliferation and apoptosis balance in the prostate cells was showed. Disturbance of this processes may lead to uncontrolled cell division and eventually to cancer development [4]. The data obtained in the *Prostate Cancer Prevention Trial* revealed that blocking the conversion of testosterone to DHT by finasteride (5α-reductase inhibitor) reduced PCa incidence. Unfortunately, prolonged use of finasteride was associated with an increased risk of other tumors and numerous side effects [12]. Further information concerning the effects of androgens on the PCa development was derived from animal models. For example, increased PCa incidence was observed in Noble rats following a long-term supply of testosterone [13]. Nobel rats were also a model in which synergistic effects of androgens and estrogens on the induction of dysplasia and hyperplasia of the glandular epithelium were demonstrated [14].

Another group of sex hormones that regulate growth and differentiation of the prostate cells and may be involved in their carcinogenesis are estrogens. The estrogens affect the prostate gland in a direct and indirect way. Indirect action is manifested by inhibition of androgen synthesis and secretion by means of estrogen influence on the hypothalamic-pituitary-gonadal axis or directly on the testes [15]. The direct mechanisms have been unveiled experimentally. It was found that estrogens, in both humans and rats, stimulated DNA synthesis and induced metaplastic lesions in the prostate epithelium [16, 17]. In rats, the presence of high doses of estrogen in the first five days postnatally triggered significant changes in the development and functioning of the prostate in later life. These changes included inhibition of the prostate growth, inflammations, epithelial hyperplasia and dysplastic lesions that histologically resembled prostatic intraepithelial neoplasia (PIN) [18].

The relationship between serum level of the steroid hormones and PCa incidence remains unclear. It is difficult to explain the increased incidence of PCa with age, when androgen bioavailability decreases. It may be speculated that the estrogens replace testosterone in the induction of neoplastic lesions, however, this assumption has not been sufficiently proved [19]. Most studies showed no relationship between the levels of steroid hormones and sex hormone binding protein (SHBP) in serum, and increased risk of PCa [20]. However, the article by Gann et al. [21] reported an enhanced risk of PCa in the patients with increased testosterone level and low level of SHBP. Unfortunately, this type of study may be burdened with serious errors due to using different laboratory methods for the determination of hormone blood levels, current endocrine status, etc. [20].

Steroidogenesis in the prostate gland

Androgens are the main type of steroid hormones with a fundamental role in the prostate growth and functioning. Their *de novo* production occurs in the testes and adrenal cortex. Peripheral tissues, such as the prostate or skin, may synthesize androgens from the precursors supplied with blood. This process depends on the expression of steroidogenic enzymes, involving different types of dehydrogenases: 3β-hydroxysteroid dehydrogenase (HSD3β), 17β-hydroxysteroid dehydrogenase (HSD17β), 3α-hydroxysteroid dehydrogenase (HSD3α) and 5α-reductase. The most important androgen is testosterone, produced from androstenedione (A-dione) in testicular Leydig cells by HSD17β3. Testosterone may be also synthesized in the prostate, however, this process is mediated by another enzyme – HSD17β5. In contrast, A-dione is formed through the conversion of an adrenal precursor dehydroepiandrosterone (DHEA), catalyzed in the prostate basal epithelial cells by HSD3β1. In the prostate testosterone is converted to its most active metabolite, dihydrotestosterone (DHT), by type 2 5α-reductase (SRD5A2) [5]. DHT level in the tissue is much higher than in blood [6]. An alternative pathway of DHT formation involves the conversion of 5α-androstan-3α, 17β-diol (3αA-diol) mediated by type 3 HSD3α. Both testosterone and DHT inactivation is catalyzed by HSD17β2. As a

result, these androgens are converted to A-dione and 5α-androstenedione (5αA-dione), respectively [5].

The prostate gland may produce not only androgens, but also estrogens. In this process, aromatase (CYP19) converts testosterone into the most active form of estrogen – 17β-estradiol (E2). A-dione may also be converted, but only into estrone (E1) that exhibits poor biological activity [7]. HSD17β 1, 7 and 12 can reduce E1 to E2. An opposite reaction is mediated by HSD17β2,4,8,10 and 11, displaying oxidative activity [8]. These mechanisms are illustrated in figure 1.

Expression of steroidogenic enzymes in the prostate cancer

Intracellular levels of steroid hormones in the peripheral tissues depend on their supply with blood and local biosynthesis and metabolism. Peripheral tissues, including the prostate gland, are not capable of *de novo* steroid synthesis, but they contain the enzymes that catalyze the formation of active androgens and estrogens from adrenal derived precursors [22]. The main enzymes involved in the local steroidogenesis in the prostate gland are steroid sulfatases, 3β-hydroxysteroid hydrogenases, 17β-hydroxysteroid dehydrogenases, 3α-hydroxysteroid dehydrogenases, 5α-reductases and aromatases [4].

Steroid sulfatases

Steroid sulfatases (STS) are responsible for the conversion of estrone sulphate (E1S) to biologically active estrone (E1) and dehydroepiandrosterone sulfate (DHEAS) to DHEA [23]. STS expression has already been investigated during studies on other hormone-dependent neoplasms, such as breast cancer and endometrial cancer [24]. It was found that breast cancer was associated with higher level of steroid sulfatase mRNA and protein, as compared to normal tissues [25] [26]. Similarly, the expression level of STS may also be important in the PCa development, considering its role in the synthesis of androgens and estrogens [27]. The studies by Nakamura et al. [28] demonstrated that estrogen production in the prostate was to a greater

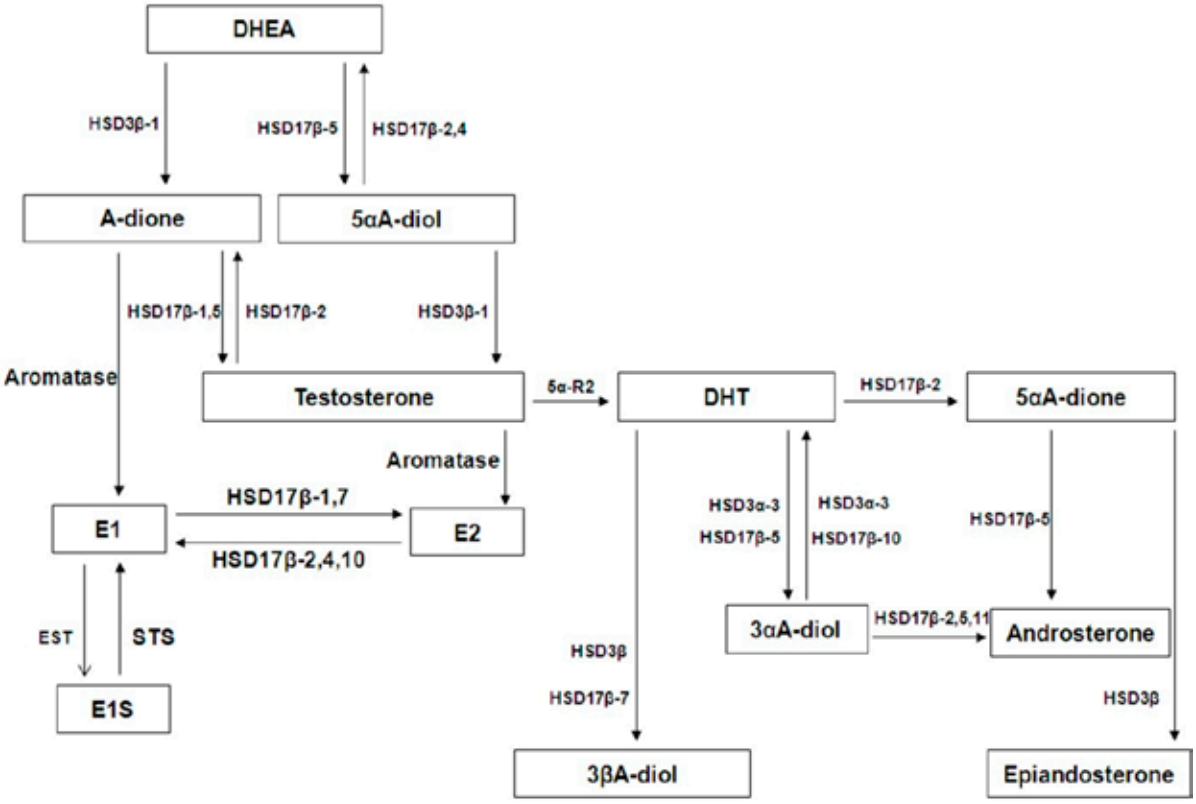

Figure 1. Steroidogenesis in the prostate gland. HSD3β1, 3β-hydroxysteroid dehydrogenase type 1; HSD17β-1, 2, 4, 5, 7, 10 and 11, different types of 17β-hydroxysteroid dehydrogenases; 5α-R2, 5α-reductase type 2, DHEA, dehydroepiandrosterone; A-dione, androstenedione; 5αA-diol, 5α-androstenediol; E1, estrone, E2, estradiol, DHT, dihydrotestosterone; 3βA-diol, 5α-androstan-3β, 17β-diol; 3αA-diol, 5α-androstan-3α, 17β-diol; 5αA-dione, 5α-androstenedione

extent mediated by STS than by CYP19. STS presence was confirmed in the majority of human neoplastic tissues, and PCa cell lines, but it was not detected in benign prostatic hyperplasia (BPH).

3β-hydroxysteroid dehydrogenases

3β-hydroxysteroid dehydrogenase (HSD3β) are bifunctional dimeric enzymes necessary in the biosynthesis of all classes of steroid hormones. There are two human isoforms of this enzyme: HSD3β1 and HSD3β2 [29]. The first isoform is expressed only in placenta and peripheral tissues, such as mammary gland, prostate gland, and skin, and the second is expressed particularly in steroidogenic tissues, such as adrenal glands, testes and ovaries, where it is involved in the conversion of dihydrotestosterone to inactive metabolites. The genes encoding these enzymes are, due to their important role in androgen metabolism, classified as candidate genes for PCa. Numerous polymorphisms have been identified in both genes and links have been found between some of them and increased PCa risk [30]. Stanbrough et al. reported overexpression of HSD3β2 gene and concluded that it was associated with a tumor adaptation to anti-androgen therapy [31].

17β-hydroxysteroid dehydrogenases

The family of 17β-hydroxysteroid dehydrogenases consists of a group of enzymes that catalyze the conversions of 17β-ketosteroids and 17β-hydroxysteroids [32]. Both estrogens and androgens display their highest activity in 17β-hydroxy form, which is present in estradiol and testosterone. Thus, HSD17β enzymes play a crucial role in the regulation of steroid hormones by controlling the formation and inactivation of androgens and estrogens. So far, at least 11 types of 17β-hydroxysteroid dehydrogenases have been characterized in different species, including 9 types in humans [33]. PCa studies revealed a differential expression of a few enzymes from 17β-hydroxysteroid dehydrogenase family, including increased expression of reducing enzymes that catalyze a conversion of precursors to active androgens and estrogens (HSD17β3, HSD17β5 and HSD17β7), and reduced expression of oxidative enzymes, catalyzing the opposite reactions (HSD17β2) [31, 34]. These data suggest a shift in androgen and estrogen metabolism within a tumor, towards the formation of testosterone, DHT, and E2. Moreover, declined expression of HSD17β2 that protects tissues against excessive synthesis of E2 may be associated with higher level of this hormone in the prostate and stimulation of cancer cell proliferation. It is important that these processes take place in the phase where PCa development is androgen-independent [35].

3α-hydroxysteroid dehydrogenases

Type 2 of 3α-hydroxysteroid dehydrogenase (3α-HSD) belongs to the family of aldo-keto reductases (AKR) and it is abbreviated as AKR1C3. In the prostate, this enzyme catalyzes conversion of Δ4-androstene-3,17-dione to testosterone, 5α-dihydrotestosterone to 5α-androstane-3α,17β-diol (3α-diol) and 3α-diol to androsterone. Thus, AKR1C3 may control the androgen balance in the prostate gland by transactivation of the androgen receptor [36]. Fung et al. [37] synthesized a highly specific antibody and used it to demonstrate cytoplasmic immunoreactivity of AKR1C3 protein in non-epithelial prostate components, such as endothelial and perineurial cells, stromal cells and smooth muscle cells. Immunoreactivity was also detected in transitional urothelial epithelium, prostatic urethra, and vas deferens epithelium. In contrast to non-epithelial cells, the glandular epithelial cells show only slight or no immunoreactivity to this highly specific antibody. Positive cytoplasmic immunoreactivity of AKR1C3 was detected in 9 of 11 cases of prostate cancer, indicating overexpression of AKR1C3 in PCa. These results confirm earlier reports on elevated level of mRNA for such reducing enzymes as AKR1C2 [38] and AKR1C3 [39] in primary cultures of prostate epithelial cells derived from prostate cancer regions. Fung et al. also reported that the second type of 3α-hydroxysteroid dehydrogenase was coexpressed with the androgen receptor in the same prostatic cell types, and particularly in the case of prostate cancer, however, this was not confirmed in immunohistochemical tests. It was found [37] that increased level of AKR1C3 might not only promote growth of the prostate cancer cells, but it also supported angiogenesis by raising the levels of VEGF [40]. Low immunoreactivity of AKR1C3 protein in normal prostatic epithelium, as compared to neoplastic tissues, was corroborated by other studies [41], which may indicate a role of this enzyme in promoting tumor growth and angiogenesis.

5α-reductases

Testosterone is a dominant circulating androgen, and it may be present in blood as a free form or as SHBP. The free testosterone fraction may diffuse into the prostate cells, where it is irreversibly converted

into dihydrotestosterone by NADPH-dependent Δ4-3-ketosteroid-5α-oxidoreductase (5α-reductase) [42]. Two types of 5α-reductase have been identified in humans, and the prostate contains almost exclusively the second type, encoded by SRD5A1 gene [43]. Results from *in vitro* studies suggest that the prostate-based conversion of testosterone to DHT, catalyzed by type 2 of 5α-reductase, may be the most important factor determining the risk of PCa [44]. SRD5A1 overexpression was found in androgene-independent prostate cancer. Enhanced expression this gene suggests intensified conversion of testosterone to its most active form – dihydrotestosterone, which may also contribute to the activation of the androgen receptor in PCa [32]. A49T mutation in 5α-reductase gene raises the enzyme activity fivefold [45]. A correlation was also revealed between SRD5A1 polymorphism and PCa risk [46]. In the Japanese population, characterized by low incidence of prostate cancer [47], the activity of 5α-reductase is much lower than in the American population, where the incidence rate is very high.

Aromatases

Prostate cells are capable of synthesizing estrogens from androgens by means of aromatase [8]. Activity of this enzyme was confirmed in prostate cancer cell lines and the cells derived from benign prostate hyperplasia, but it was not detected in normal epithelium. This indicates a possibility of local conversion of testosterone to estrogens that occurs in PCa, which may eventually result in intratumoral increase of estrogen levels [48]. It was also showed that obesity, which is most often associated with elevated androgen aromatization, was correlated with poor histology in young PCa men, particularly those younger than 50 years [49]. Prostate tumorigenesis involves changes in the cellular expression of aromatase. It is believed that this may be related to the development and progress of PCa, in a mechanism similar to the one described earlier for the breast cancer [50]. Inhibited estrogen biosynthesis in aromatase knockout mice led to the prostate enlargement, but no signs of a malignant transformation were observed [51]. There are also reports in which aromatase expression was detected in neither benign prostatic hyperplasia, nor prostate cancer cell lines [52], and therefore the role of this enzyme in PCa development remains unclear. Polymorphisms of aromatase encoding *CYP19* may also play an important role in the development of prostate cancer [53], however, conclusive evidence is lacking due to numerous limitations of such studies.

Diagnostic potential

Early and accurate diagnosis is a critical issue for subsequent treatment of PCa. Unfortunately, common diagnostic tools have some disadvantages and in many cases are not sufficient for proper PCa recognition. The standard test used for prostate-specific antigen (PSA) amount in a blood has multiple limitations e.g. not distinguishing well between BPH and PCa and having a normal PSA levels does not completely rule out the cancer [53]. Similar problems can occur during Digital rectal examination (DRE) which is also used for PCa detection. Occasionally men with normal DRE results suffer from PCa. In opposite, men with an abnormal PSA level or positive DRE, diagnosed and treated for PCa, would have never develop disease to advanced stage. Biopsy is considered as accurate and direct way for an indication of tumor cells presence in the gland, however, collecting tissues from the small area raises the possibility that cancer can be simply missed [54]. Some of these disadvantages can be overcome by the introduction of new prognostic tests based on genes expression. Prolaris is the new diagnostic test for PCa which measures the expression level of genes responsible for cancer cells division [55]. Some other tests like: Oncotype DX, measure the expression level of various genes implicated in PCa [56]. These tests are used to provide an additional information in predicting disease aggressiveness. In cases when the first biopsy result was negative and patient has still an elevated level of PSA, decision about the next biopsy can be make on the basis of new Hologic [57], MDxHealth [58] and Mitomics [59] tests. During prostate carcinogenesis an altered expression of genes encoding steroidogenic enzymes can occur. This abnormalities can be a potential source for new diagnostic tools development as well as for a choosing of the most effective therapy. However, better understanding of abnormal genes expression and the role of steroid hormones in prostate carcinogenesis is needed.

Clinical applications

Androgen-deprivation therapy (ADT) remains the principal treatment for advanced and metastatic PCa. Androgen withdraw can be achieved with gonadotropin-releasing hormone analogs or anti-androgen agents. Treatment with gonadotropin-releasing hormone analogs like leuprolide or goserelin is called chemical castration due to the fact they lower androgens level, whereas anti-androgens (e.g. flutamide or bicalut-

amide) directly block the androgen receptor. The benefits of ADT include disease remission, symptom reduction and a marked clinical response. Decrease in testosterone plasma level results in apoptosis of most BPH as well as PCa cells. However, after an initial response to ADT, disease progress occur regardless of testosterone level. Somehow, tumor cells become able to maintain the transcription of androgen related genes. This stage of cancer is known as castrate-resistant prostate cancer (CRPC) and is associated with a poor prognosis and short survival. Mean survival time for metastatic CRPC is only in range of 16-18 months [60, 61]. Some evidence [62, 63] suggest, that up-regulation of genes encoding enzymes involved in androgen biosynthesis such as AKR1C3 can be key event in tumor adaptation to ADT. Moreover, other studies [64, 65] suggest PCa bone metastases ability to conversion of adrenal androgens to testosterone and DHT. Due to these facts, steroidogenic enzymes can be important target for the development of new therapies for PCa based on the use of their analogs and inhibitors. In recent years new strategies to inhibit AR signaling have been emerge. It was shown, that treatment with cytochrome P450-c17 (CYP17) inhibitor, abiraterone acetate, results in suppression of adrenal derived androgens. It was associated with increased survival time and increased time to PSA progression [66]. Other drug with anti-androgen effect is ketoconazole that blocks P450s involved in ergosterol biosynthesis [67].

In light of these observations, steroidogenic enzymes inhibition has high potential value in CRPC treatment however certain conclusions required more time and researches.

Conclusions

Steroid hormones participate in the regulation of growth and functioning of the prostate gland and its pathogenesis. The amount of steroid hormones in the tissue depends on blood supply and local activity of steroidogenic enzymes responsible for their biosynthesis and inactivation.

Scientific studies published so far have indicated a shift in the steroidogenic enzymes expression pattern towards the synthesis of active forms of androgens and estrogens during prostate carcinogenesis and androgene-independent tumor growth phase. This process may be greatly influenced by polymorphisms of genes encoding the steroidogenic enzymes. Unfortunately, there are too few publications to draw ultimate conclusions. Identification of altered expression of the steroidogenic enzymes during neoplastic transformation of the prostate gland may provide valuable information on the mechanisms regulating this process. This knowledge may be extremely useful for the development of new therapies for prostate cancer, based on the use of analogs and inhibitors of the steroidogenic enzymes. However, it is still difficult to demonstrate a direct contribution of individual factors to the initiation and progression of PCa. More data is needed to fully understand the phenomena occurring during the prostate carcinogenesis.

Conflict of interest

The authors declare that there are no conflicts of interest.

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CASE REPORT

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Microangiopathic antiphospholipid syndrome (MAPS) in the course of undifferentiated connective tissue disease

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ABSTRACT

Background. The microangiopathic antiphospholipid syndrome (MAPS) is a subset of APS comprising those patients presenting with thrombotic microangiopathy and demonstrable antiphospholipid antibodies. Renal involvement occurs frequently in the course of MAPS with clinical symptoms of acute renal failure, hypertension, proteinuria and erythrocyturia.
Case report. The report presents the patient with MAPS confirmed by kidney biopsy in the course of undifferentiated connective tissue disease.
Conclusion. The authors emphasize the importance of effective anticoagulant treatment in order to inhibit thrombosis and renal damage in the course of MAPS.

Key words: microangiopathic antiphospholipid syndrome, connective tissue diseases, chronic kidney disease, anticoagulant treatment.

Background

The Antiphospholipid Syndrome (APS) is characterised with recurrent venous and/or arterial thrombosis, obstetric complications and the presence of antiphospholipid antibodies (aPLs) [1]. The APS is considered to be one of the most common systemic connective tissue diseases, as a syndrome with primary character or occurring in the context of other autoimmune diseases (secondary APS) [2]. In 2006 it was proposed that the subset of microangiopathic antiphospholipid syndrome (MAPS) be distinguished, comprising the cases of thrombotic microangiopathy (TMA) with the presence of aPLs [3].

The aim of this paper is to present the case of a patient with MAPS in the course of undifferentiated connective tissue disease.

Case report

A 51-year-old male patient was first subjected to examination in the Department of Rheumatology and Connective Tissue Diseases in Lublin in July 2009. According to the data obtained from the patient the first symptoms of the disease appeared in February 2008 in the form of brown skin discolorations on his back, then on the skin of chest, abdomen, upper and lower extremities associated with increasing weakness. The patient was not treated before for any cause, he had been a smoker for ca 30 years (15/day on average). The results of the outpatient laboratory tests conducted in May 2008 confirmed anaemia and elevated ESR level. No significant pathology was found in the skin and lymph node biopsy or in serological tests for viral diseases. Since September 2008 the patient experienced subfebrile body temperatures with periodic fever ⩽ 39^0C with good response to antipyretics; permanent fever since December 2008. Since October 2008 the elevated arterial blood pressure was observed; since December 2008 body weight loss (3 kg in 2 months), night sweats and cough; since January 2009 elevated serum creatinine and proteinuria. In February 2009 during the business trip abroad the patient experienced significant worsening of his condition with constant fever > 39^0C, hypertension (AH) (systolic > 220 mmHg), fine-spotted trunk rash and arthralgia (mainly knee and hip joints). The patient was admitted to hospital, Department of Rheumatology

and Clinical Immunology where the test results showed anaemia (haemoglobin 9.0- 8.2 g/dl) with decreased haptoglobin level (8 mg/dl) and normal levels of bilirubin and platelets, leukocytosis (164000/ul), increasing renal function impairment (creatinine 2.2- 3.6 mg/dl, urea 75-138 mg/dl, cystatin C 3.01- 4.44 mg/l, estimated glomerular filtration (GFR) 34.5–21.9 ml/min/1,73m^2), proteinuria and erythrocyturia, elevated concentration of lactate dehydrogenase (LDH) 319–414 U/l and CRP (13,5 mg/dl), decreased complement component C3 (54 mg/dl). The serological tests were negative for: antinuclear antibodies (ANA), anti-cyclic citrullinated peptide (anti-CCP) antibodies, rheumatoid factor (RF), antineutrophil cytoplasmatic antibodys (ANCA); the presence of anti-SS-A/Ro antibodies was detected. The hemolytic-uremic syndrome (HUS) was suspected, for differentiation from thrombotic thrombocytopenic purpura (TTP). The treatment for arterial hypertension (amlodipine and valsartan) and with glucocorticosteroids (GCS) [intravenous (iv) prednisolone at a total dose of 1000 mg followed by oral prednisolone in the dose of 40- 30 mg] was initiated, with which the fever subsided permanently and the articular complaints decreased; packed red blood cells were transfused. The ADAMTS-13 protease activity was determined as 34% (normal range: 45- 110%) without confirming the low (<5%) enzyme activity typical for TTP. Due to persistent symptoms of haemolysis and increasing renal insufficiency the patient was transferred to the Department of Haematology and then to the Department of Kidney Diseases, Hypertension and Rheumatology. The renal biopsy was performed, in the histopatological examination of the biopunctate the features of thrombotic microangiopathy (TMA), diffuse ischemic damage of renal glomeruli, acute tubular necrosis and renal interstitial fibrosis were described; no deposition of immune complexes or complement was detected. The Doppler ultrasound imaging of the renal blood vessels did not confirm their stenosis. Given the high levels of arterial hypertension, symptoms of over-hydration and increasing renal insufficiency (creatinine 6.4–8.0 mg/dl, urea 143–231 mg/dl, eGFR 9.8–7.6 ml/min/1.73m^2), haemodialysotherapy was initiated. The primary suspicion of HUS and TTP was rejected on the basis of mild haemolysis, absence of schistocytes, thrombocytopenia and low ADAMTS-13 levels. The diagnosis of TMA in the course of malignant hypertension or potentially in the course of previously undiagnosed systemic sclerosis (skin changes, narrow lips were described) with renal crisis was made. The treatment with GCS was maintained (prednisolone 20 mg/d, at the attempt of discontinuation the recurrence of fever and joint pain was observed), the treatment for hypertension established (valsartan 160 mg/d, torasemide 100 mg/d, amlodipine 10 mg/d, metoprolol 95 mg/d).

During the patient's treatment in the Department of Rheumatology in Lublin since July 2009 the following were observed: in the physical examination, face skin hardening with the features of microstomia, numerous telangiectasias on the skin of face, chest; oliguria (diurnal urine 500 ml), arterial hypertension. The immunological tests confirmed the presence of anti-Ro52 and ANA antibodies with granular fluorescence pattern (titre: 1:160) and IgG anti-β_2 glycoprotein-I (anti-β_2GP-I) antibodies (46.55 SGU, positive result > 20). The presence of ANCA, anticardiolipin (aCL) antibodies, lupus anticoagulant (LAC) was not detected. In the contrast examination of the upper gastrointestinal tract the esophageal motility abnormalities were not found. The capillaroscopy demonstrated slight disorganisation of microvascular array, however without the features of scleroderma microangiopathy. In the high-resolution computer tomography (HRCT) of the chest no interstitial changes or features of pulmonary embolism were found. The pulmonary function tests showed the preserved diffusion capacity and lung volumes. In the ECG, slight reduction of the ejection fraction (59 %) and increased pulmonary artery pressure (PASP 37 mmHg) were observed. In the salivary gland scintigraphy the functional disorders of submandibular glands were observed; negative Schirmer's test.

Based on data collected from the medical history and the results of biochemistry, serological, histopathological (including kidney biopsy) and imaging tests the thrombotic microangiopathy (TMA) was diagnosed which in the presence of anti-GP-I antibodies has the form of the microangiopathic antiphospholipid syndrome (MAPS) in the course of undifferentiated connective tissue disease.

The decision was taken on the introduction of anticoagulant therapy with an oral anticoagulant (warfarin) and with the inhibitor of platelet aggregation (clopidogrel). The dose of GCSs was reduced (prednisone 5 mg/d, since February 2010 2.5 mg/d) and the cyclophosphamide therapy was initiated (400 mg in intravenous infusions every 3–4 weeks; then after the total dose achieved 5800 mg, every three months). In the treatment for arterial hypertension and as a cardiovascular protective therapy, angiotensin convertase inhibitors were introduced (enalapril 25–40 mg/d, with partial replacement with cilazapril due to dry cough), amlodipine was replaced with verapamile 160 mg/d, and statin was added (simvastatin 20 mg/d).

As the result of the applied treatment the proper control of the arterial hypertension, permanent diure-

sis (2000–3000 ml/d) and stabilisation of the renal function with reduced frequency of haemodialysis, less severe skin changes were obtained.

Discussion

The case presented herein illustrates the problem of early diagnosis and initiation of treatment for MAPS developing in the course of undifferentiated connective tissue disease. The thrombotic microangiopathic changes described in the histopatologic examination of kidney biopunctate together with the presence of anti-β_2GP-I antibodies allow making the MAPS diagnosis. The early initiation of anticoagulant therapy would have probably enabled the inhibition of the thrombotic process and maintaining the renal function.

The thrombotic process in the course of APS often involves kidneys and the clinical symptoms depend on the renal vascular calibre and activity of the thrombotic process [2, 4–5]. One of the forms of renal involvement in the course of APS is the APS nephropathy resulting from the thrombotic microangiopathic changes in small renal blood vessels and glomeruli with the absence of immune deposits or inflammatory cells (small vessel occlusive disease) [2, 4–5]. The symptoms of APS nephropathy include arterial hypertension (in nearly all patients), reduced GFR with potential acute renal failure, proteinuria and erythrocyturia. The best diagnostic method is the renal biopsy; it is, however, rarely performed due to the concomitant anticoagulant therapy [2, 4–5].

The thrombosis of small blood vessels (e.g. in kidney, retina, skin, lungs, liver or intestine) is the vital part of the clinical picture of APS. In 2006 the subset of APS termed MAPS was distinguished; it covers the cases of classic primary autoimmune diseases (e.g. systemic lupus) with TMA, but without the obstruction of large blood vessels [3, 6–9]. The MAPSs include also thrombotic thrombocytopenic purpura (TTP), hemolytic-uremic syndrome (HUS), HELLP obstetric syndrome, catastrophic APS (CAPS), provided that the symptoms of large blood vessel obstruction do not appear [2–3, 6–9]. These are syndromes where the TMA features are found in the microcirculation in various organs and the occurrence of aPLs is observed, probably induced by the damage of the blood vessel endothelium [7]. The symptoms in such patients appear upon the activation of usually similar factors (infections, drugs, pregnancy) and are similar (haemolytic anaemia, thrombocytopenia, acute renal failure).

The MAPS management is conducted on the basis of scarce experience from clinical observations given the lack of international guidelines [4]. It is based on the successful lifelong treatment for arterial hypertension and anticoagulant therapy, aiming at stopping the process of renal failure [2, 5]. The treatment depends also on finding whether the MAPS occurs in the course of primary APS or another autoimmune disease. In the case of systemic disease, the treatment based on GCSs or complementary immunosuppressive therapy may be indicated, depending on the activity of primary disease [2, 4]. It has been suggested recently that the aPLs have pathogenic effect not only on the haemostatic system but also activate the complement system and increase the inflammatory condition leading to tissue damage [4, 10]. For these reasons, getting under control simultaneously the thrombotic and inflammatory process seems to be of particular advantage for these APS patients [4].

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Prospective observational study on predicting adverse clinical outcomes in patients with implanted defibrillating devices – a study rationale, design and principal methods

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ABSTRACT

The project "Predicting adverse clinical outcomes in patients with implanted defibrillating devices" is a prospective observational study in a group of high risk cardiac patients with already implanted either an implantable cardioverter-defibrillator (ICD) or a cardiac resynchronization therapy device with defibrillating mode (CRT-D). The primary project aim is to build predictive models for an appropriate ICD or CRT-D interventions and other adverse clinical outcomes based on clinical assessment of the cardiovascular system, general clinical status, advanced mathematical and statistical analysis of cardiovascular time series and biological signals, including pressure waveforms, ECG and cardiac impedance. Up to 600 ambulatory adult patients are scheduled to be enrolled and after the first visit they are expected to be followed-up every 6 months for a number of clinical outcomes, including antiarrhythmic therapy intervention, sudden and aborted cardiac death and total mortality. The project launched in March 2010 and is expected to end in February 2014.

Key words: cardiac resynchronization therapy; implantable cardioverter-defibrillator; risk prediction; sudden cardiac death; ventricular arrhythmia.

General information

The project "Predicting adverse clinical outcomes in patients with implanted defibrillating devices" was awarded a research grant from the Foundation for Polish Science from Warsaw, Poland, in the 4th edition of the TEAM competition in February 2010. The contract between the Foundation for Polish Science (FPS) and Poznan University of Medical Sciences (PUMS) in Poznan, Poland, was signed on the 1st of March 2010, and the duration of the project was planned for 48 months, until the 28th of February 2014.

This project is a prospective observational study in a group of high risk cardiac patients with already implanted either an implantable cardioverter-defibrillator (ICD) or a cardiac resynchronization therapy device with defibrillating mode (CRT-D). The patients should have survived a myocardial infarction and/or suffered from chronic heart failure with severely depressed left ventricular (LV) function with ejection fraction (LVEF) of no more than 35% at the time of ICD or CRT-D implantation, or be survivors of sudden cardiac arrest with implanted ICD or CRT-D, or suffer from cardiac diseases with an increased risk of premature death like hypertrophic cardiomyopathy, LQT syndrome or Brugada syndrome. The project addresses the field of electrotherapy with implantable cardioverter-defibrillators (ICD or CRT-D) which main (ICD) or one of main (CRT-D) functions is the application of electrotherapy for the treatment of life threatening ventricular arrhythmias like ventricular tachycardia (VF) or ventricular fibrillation (VF). The electrotherapy is applied in a form of antitachycardia pacing that is faster than VT or energy discharge

by intrathoracic defibrillating electrode to stop VT/VF. The project is interdisciplinary in nature and requires a close co-operation of specialists in medicine, engineering, software development, and basic science.

Management

Principal Investigator: Associate Professor Przemyslaw Guzik from PUMS.

Co-investigators: Associate Professor Jaroslaw Piskorski from University of Zielona Gora in Poland, Professor Andrzej Wykretowicz from PUMS and Professor Henryk Wysocki from PUMS.

International partners: Professor Georg Schmidt from Klinikum rechts der Isar, 1. Medizinische Klinik, Technische Universitat Munchen, Munich, Germany; Professor Adrian Baranchuk from Department of Cardiac Electrophysiology and Pacing at the Kingston General Hospital of the Queen's University in Kingston, ON, Canada; Professor Wojciech Zareba from Heart Research Centre, University of Rochester Medical Center, Rochester, NY, USA; Professor Marek Malik Cardiac Electrophysiology at the University of London, Head of Non-Invasive Electrophysiology unit at St. George's, London, UK.

Ethics

Bioethical Committee at Poznan University of Medical Sciences on 10th April 2010 accepted all project's protocols and forms, including information for patients and the form of their consent for study participation and withdrawal. The permission number is 363/10.

Finance

The total value of granted funds is 1,520,000.00 Polish Zloty which at the time of contract signing amounted to nearly 390,000.00 Euro. The funds were designed for research stipends for 3 medical students, 3 PhD students and one post-doc – all of them had to be recruited from open calls. Additionally, funds were earmarked to purchase new equipment, tools and software, cover the costs of biochemical analysis, domestic and international exchange, promotion of study results and publications as well as for work remuneration.

Research Basic Concept

Devices such as ICD and CRT-D save lives in high risk cardiac conditions like post-infarction, non-ischemic heart failure, in survivors of sudden cardiac death secondary to VT/VF, hypertrophic cardiomyopathy, long QT syndrome or Brugada syndrome [1–3]. There are thousands of people with implanted defibrillating devices worldwide and all these patients have created a new clinical population since ICD and CRT-D have been implanted. Only a minority of such patients have appropriate life-saving intervention and for this reason the numbers of patients needed to treat in order to save one life is quite high. Based on the established criteria for ICD/CRT-D implantation, it is necessary to implant an ICD or CRT-D to several patients to save one life – the number needed to treat patients with such a device depends on the duration of follow up [1–3]. Based on the MADIT II trial results this number was 133 after 1 year, and substantially dropped to 17 after 2 years and to 8 the end of a 3-year follow-up [4–6]. These figures show that early criteria proposed for ICRD and CRT-D implantation before 2010, when this project was launched, for the identification of high risk patients, were not effective.

Numbers of studies attempted to establish optimal systems of identification high-risk patients. These studies, however, compared ICD/CRT-D with placebo or pharmacological antiarrhythmic therapy or CRT-D vs CRT alone (CRT without defibrillating mode). However, before 2010 not a single prospective study focused on patients with already implanted defibrillating devices. Our study hypothesis was that it is possible to improve the identification of high-risk patients eligible for ICD or CRT-D implantation based on a non-invasive assessment of the cardiovascular status and general patient condition.

Research Project Objectives

The study primary objective is to build various predictive models for an appropriate ICD or CRT-D interventions and other adverse clinical outcomes. The models will be based on both detailed and wide clinical assessment of the cardiovascular system, general clinical status, and modern advanced mathematical and statistical analysis for cardiovascular time series and biological signals as pressure waveforms, ECG and cardiac impedance waves. The study secondary objective is to develop software and tools for the analysis of the variability and asymmetry of cardiovascular time series, for the detailed analysis of the ECG structure and arterial pressure waveforms mechanical properties.

Research Plan

Ambulatory patients with already implanted ICD or CRT-D and who fulfill the project's inclusion criteria (Table

1) will undergo a detailed non-invasive clinical examination with biochemical analysis of their venous blood and urine samples. Patients will be followed-up every 6 months during their routine visits at our out-patient clinic or, in case of a missing visit, by phone or mail. During each visit the current status of the implanted device will be routinely checked by telemetry and all events (arrhythmias, interventions), if recorded by the device, will be transferred and stored for further analysis.

Research methodology

Study population

Up to 600 patients after an elective implantation of ICD or CRT-D, which were in agreement with the most current guidelines and recommendations for an implantation of a respective device. Summary of eligible patients is shown in Table 1.

Methods

All patients will undergo the following methodological approach:

- history taking, physical examination and anthropometrics, including body composition analysis;
- functional cardiovascular evaluation with the 4-degree New York Heart Association functional class, echocardiography, the 6-minute walking test, cardiac impedance, arterial photoplethysmography and applanation tonometry [7–15];
- structural cardiovascular evaluation of carotid arteries, aorta and the heart with ultrasound methods [8, 9, 11, 13];
- measurement of indices of autonomic modulation of the cardiovascular system, and cardiovascular variability, including variability and asymmetry of haemodynamics parameters, heart rate and blood

Table 1. Eligibility and inclusion criteria for patients enrolled to the project "Predicting adverse clinical outcomes in patients with implanted defibrillating devices"

ELIGIBILITY AND INCLUSION CRITERIA
Patients with an already implanted ICD or CRT-D device for the following clinical indications: – post-infarction patients with LVEF <35 % at the time of implantation; – patients with chronic heart failure on an optimal pharmacological treatment, QRS duration >130 ms and functional dysfunction (NYHA III or IV) and LVEF < 35% at the time of implantation; – survivors of sudden cardiac arrest or aborted sudden cardiac death in a mode of ventricular tachycardia or ventricular fibrillation; – patients with hypertrophic cardiomyopathy; – patients with Brugada syndrome; – patients with LQT syndrome; – patients with right ventricular arrhythmic cardiomyopathy.
Patients who underwent an implantation of ICD or CRT-D at least 1 month earlier.
Ambulatory patients who do not require hospitalization due to their haemodynamic status.
Patients who will give their informed consent to participate in the study, both in the enrolment and later follow-up.

Table 2. Clinical outcomes of the project

PRIMARY OUTCOMES
1. Mortality of any cause; 2. Fatal sudden cardiac death, aborted sudden cardiac death or sudden cardiac arrest; 3. Appropriate antiarrhythmic therapy of discharge of the ICD or CRT-D; 4. Combination of 2 & 3; 5. Combination of 1 & 3.
SECONDARY OUTCOMES
1. Hospitalization due to advancing heart failure; 2. Death related to heart failure aggravation; 3. Non-cardiac death; 4. Recurrent arrhythmic events defined as: an appropriate antiarrhythmic therapy of discharge of the ICD or CRT-D, or aborted sudden cardiac death, or sudden cardiac arrest; 5. Electrical storm defined as at least 3 appropriate antiarrhythmic therapy of discharge of ICD or CRT-D during 24 hours with at least 1 minute gap between each; 6. Syncope of any cause; 7. Syncope of an unknown cause; 8. Combination of fatal sudden cardiac death, aborted sudden cardiac death or sudden cardiac arrest or an appropriate antiarrhythmic therapy of discharge of ICD or CRT-D, or syncope of an unknown cause.

pressure variability and asymmetry, arterial baroreflex function [16–27];
– biochemistry of fasting blood and urine samples for NT-pro-BNP (N-terminal prohormone of brain natriurectic peptide) and standard clinical biochemical parameters [28–29].

All data will be collected in the dedicated database-analytical system Granary (www.OpenGranary.com, version 2.3, Poland).

Statistical analysis – basic and advanced for building multivariate models predicting an appropriate antiarrhythmic intervention and other clinical outcomes defined in Table 2.

Expected results

According to existing publications we expect that some of the variables like very low left ventricular ejection fraction, increased end-diastolic diameter of the left ventricle, reduced deceleration capacity, the distance covered during the 6-minute walking test, the increased C-reactive protein, NT-pro-BNP, creatinine or mean resting heart rate will have a significant association with adverse clinical outcomes. However, in this study we plan to analyse many more variables which have never been studied in patients with implanted defibrillating devices. Moreover, we intend to build several predictive models starting from the simplest possible, to the minimal number of included variables, the cheapest to put into practice and ending with the most sophisticated and most precise in identification of high risk patients. All of the models will be published and available for free in a variety of ways, like e.g. stand-alone programs or Excel macros. We hope that with the obtained models we will be able to identify more precisely than it is possible now the high-risk patients who need an extra medical attention and treatment like invasive electrophysiological ablation of the heart. We also hope that with the identification of low-risk patients it will be easier to re-define some indications for ICD or CRT-D implantations and thus, finally, further reduce the costs of implantation procedures.

We plan to develop software for the analysis of the variability and asymmetry of various cardiovascular time series like heart rate, blood pressure, stroke volume, cardiac output or vascular resistance. We also intend to develop another software for the pressure waveform analysis and check the clinical value of obtained quantitative parameters. Finally, we want to develop the software for a detailed analysis of digital ECG and apply mathematical, physical and statistical algorithms to study its clinical utility.

We also assume that with the collected data from all patients with huge number of parameters and outcomes we will built our own database for big data analysis.

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PRO MEMORIAM

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Children and youth in forensic-medical views of Edmund Chróścielewski[1] (1914–1998) Recollections on the centenary of his birthday

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What can link forensic medicine with pediatrics? Certainly sudden, unexpected death of a child and the indispensable post-mortem examination. Anything more than thanatology? Professor Edmund Chróścielewski (Figure 1), the multi-year head of the Chair and Department of Forensic Medicine, School of Medicine in Poznań (1952–1985), whose centenary of birthday elapses in 2014, provided an example that the broad problems of children and youth may be within the apparently distinct scope of interest manifested by a forensic medic.

This aspect of Professor's professional activity remains unknown to paediatrists, even within their Poznań community. The centenary of his birthday provides an excellent chance to recall this significant portion of Professor's studies and opinions. Also problems of Polish children and youth under occupation and war were always in his interests. As a prisoner o the concentration camp in Auschwitz and a participant in the active struggle with the occupant he carried his personal painful experiences.

As a specialist in forensic medicine and pathomorphology, in the early 1950th he devoted several of his investigations to problems of post-mortem examination of children with inborn developmental errors. They included diaphragmatic hernias, oesophageal atresia, organic heart diseases. On the basis of pathomorphological patterns he was undertaking attempts of clarifying causes of death in newborns deceased in the course of erythroblastosis and kernicterus. He conducted also forensic evaluation of pulmonary atelectasis in newborns [1]. Problems of juvenile age toxicology [2] and forensic evaluation of foetuses were were within his interests. An interesting casuistic publication, of which he was a co-author, involved analysis of group

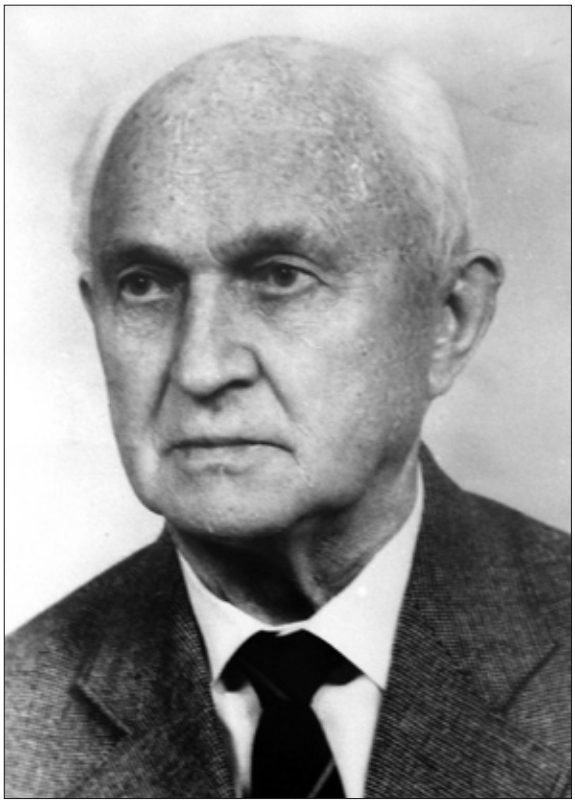

Figure 1. Professor Edmund Chróścielewski

[1] Edmund Chróścielewski (1914–1998), professor, head of the Chair and Department of Forensic Medicine, Medical School in Poznań (1952–1985), political prisoner in concentration camp of Auschwitz, a soldier of the Home Army, participant of Warsaw Uprising, author of approximately 200 publications, i.a. within scopes of forensic medicine, social problems, war and occupation, including martyrology of Polish children, participant in identification of bodies in Katyń graves, multi-year guardian of the University Sports Club (AZS).

intoxication with anilin, contained in an ink used for labelling clothes [3].

The undoubtful exponent of his experience involved the first in Poland textbook on „Autopsy of foetal and newborns' bodies" [4], prepared in co-operation with Halina Szperl-Seyfriedowa, which was published also in Russian. In co-operation with pathomorphologists (J. Groniowski, M. Rożynek) he analysed causes of death in foetuses and newborns.

He documented and analysed causes of death in children following, e.g. treatment with oxytetracyclin [5] or following intramuscular administration of debecylin, streptomycin with novocain [6]. As indicated by the conducted analysis, the death developed due to anaphylactic shock, induced by administration of novocain. In co-operation with T. Marcinkowski he published a book on „Lethal complications following application of antibiotics" (1964). In co-operation with a lawyer and a psychiatrist he analysed the problem of infanticides [7]. He published also in German and French languages.

Professor Chróścielewski, perhaps as the first forensic medic, already in 1960s stressed the social role of forensic medicine [8]. In the forensic-medical aspect he evaluated problems of traffic safety and the problem of pharmacological doping in sport [9]. The problems were particularly familiar to him due to his direct contact with sport activity among the youth: for 20 years he fulfilled the role of guardian of the university's Academic Sports Association (AZS).

His own forensic experience in obstetrical problems (abortion, delivery and puerperium) and his interest in infanticide problems resulted in chapters devoted to such subjects in a textbook of forensic medicine [10].

Together with Kazimiera Brodzińska he drew readers' attention to difficulties in diagnosing death in cases of hypothermia [11], particularly in children. He concluded that in such cases criteria of conducted resuscitation and, in particular, qualification for taking organs for transplantation should be particularly demanding.

A separate problem, to which he devoted a lot of attention, involved „Children and youth in years of the Second World War" [12]. He analysed and presented the scope of martyrdom experienced by Polish children during Nazi occupation. In his works he demonstrated that biological extermination of Polish children and youth involved a programme prepared in the smallest details. The extermination was expressed in stripping Polish inhabitants, including children, of the elementary human rights. In the areas included to the Reich a list of first names was prepared, which can be given to Polish newborns, Polish schools were closed, it was forbidden to attend theatres, museums and libraries, rigorous food rations were formulated. Children and youth of 14 to 21 years of age were transplaced to perform compulsory work in Germany or in the place of inhabitance (the so called Baudients), frequently for over 12 hours a day. Moreover, a systematic germanizing activity was conducted on Polish children. The discovered documents illustrate „robbery" of Polish children, particularly those evaluated as „racially valuable". In line with Himmler's plan and that of the Office for Racial Policy, NSDAP all care and educational institutions were taken into supervision, forbidding use of Polish language. Children originating from mixed families, devoid of parents (arrested or deceased ones) or those under care of other family members were taken to German educational institutions (among other in Poznan, Puszczykowo and Kalisz) or to German substitute families. In order to obliterate traces of the felonious activity, date of birth, first and second names of the children were altered to German ones (the institution of „Lebensborn" E.V.). Chróścielewski [13] indicated that such individual actions were less known. On the other hand, everybody knew on the mass expatriation of Zamojszczyzna inhabitants in 1943, including around 30 thousand children, of whom 4450 following „racial" examination were sent to the Reich to become Germanized. Many children died in special camps with aggravated restrictions, i.a. in Łódź in which „Polen Jugendverwahrlager der Sicherheitspolizei in Litzmanstadt" was active. Beginning at the 8th year of age the children were obliged to work. Failure to execute obligations was punished by deprivation of food, arrest or flogging. Emaciating diseases which ended in death of children were widespread. Approximately 12 thousands children passed through the Litzmanstadt camp, during liberation 600 to 800 children were saved but a severely ill proportion of them died away. Children wards were functioning also in Majdanek and Auschwitz concentration camps [13]. Jewish children were subjected to full extermination, Today it is hard to believe that at a medical meeting of physicians (Matthias Mayer, Inowroclaw, 22nd July, 1944) the opinion was expressed that "Polish children should not be treated, their therapy is not necessary and not required by the national-socialistic policy of Germany" (after 12). Professor Chróścielewski explicitly stressed that "crime on innocent children represents one of the darkest pages of the history of the Second World War. It represents a disgrace of 20th century [18]. His contemporary from the same university, the paediatri-

cian professor Olech Szczepski expressed the opinion that crimes of the years, performed in concentration camps of Auschwitz, Dachau and Buchenwald stripped physicians of their moral immunity" [19]. The relevant group of Chróścielewski's publications recalled the events painful but historically important for the Polish nation, particularly significant in view of the sporadically appearing pro-nazi slogans.

The vision of a social role manifested by forensic medicine led to a multidirectional development of the problems range by Chróścielewski, particularly as related to youth. He analysed relationships of youth drowning with alcohol consumption, increasingly severe problem of traumas [14] and of suicides among the youth [15]. In collaboration with paediatricians, i.a. at the cycle of conferences devoted to "Pubescence period" he discussed the effect of civilization on development of negative attitudes among the youth [16]. In his opinion the negative phenomena developing among the youngsters reflect inability of parents and caretakers to transmit the system of values and to demonstrate positive social sense of life. He presented his own view and interpretation of social conditioning behind the youngsters' movement of "hippies"[2] and "git-men"[3] [17]. He indicated that deep social changes lead to "weakening of family bonds and reduction of family role in socialization of the new generation"... "the youth becomes an increasingly distinct sociological category with its own problems and own position in the social structure". Continued stating that "means of mass communication easier reach the youth than contents transmitted by their direct educators...and they offer patterns and descriptions of violence, atrocities and degenerated sex." The tendency for shaping the independent youth world is promoted by advertising employing youth slang. "The fashion of "youth style" or "youth music" brings enormous income. Scepticism, egoism and consumer's approach to life, lack of ideals, indifference toward several problems basic to the society characterize youth of the sceptical generation". He continued to conclude that " a dramatic clash developed between the pattern of social reality and the system of noble principles and high ideas transmitted to the young individuals".

A phenomenon has also appeared of "rejecting any symbols which people used to value". Multiple behaviours of young people were calculated just to shock the surroundings. " The rules of crowd psychology changed street manifestations into street rows. Stones, bottles, pavement blocks became the weapons commonly used by the rebellious in their fights with the police". ..."in highly developed industrial civilizations positive phenomena are manifested in parallel with negative problems, reaching the range of social pathology, social maladjustment involving behaviour inconsistent with moral norms: alcoholism, prostitution, vagrancy, drug abuse down to criminal activities: downright robbery, hoolighanism, sexual offences, etc". This text originated from 40 years ago. The problems of contemporary young people perhaps are not foreign nowadays, they just function under another heading. Despite the elapsing multiple years his appeal that "sexual education should precede street education" [8] continues to be up-to-date.

The problems dealt with by E. Chróścielewski expressed his particular care for the life path selection by the Polish youth. It should not be forgotten that his "passion involved the youth" [18]. He represented a commonly valued educator and friend. In 1958–1964 he organized and directed social-educational-recreation camps for students of medicine of his university. Not a single sport event at the university elapsed without his participation [20]. His war experiences (arrest by Gestapo and stay in concentration camp) left an indelible trace in his psyche and directed his interest of a forensic medic [21]. At his initiative in 1968 University Council of the Polish Students' Association in Poznan convened a national symposium devoted to martyrdom of children at the area of previous Warthegau (the region of Warta river), during which he presented an appropriate lecture. He enjoyed having many talents: he wrote poems, made drawings. Few of his drawings

[2] "Hippies" or "flower-children": youth of a narrow social margin, shocking their environment by their behaviour and the passed to pathological limits disregard to the commonly established norms of social life. In their refuge from society, the group attempts to help themselves using drugs, in their attempts of rationalizing their behaviour they reach to philosophy of buddhism and ceremonies of oriental cultures. In the programme of Polish hippies one can cote elements of ideological and political diversion and, occasionally, inspiration for undertaking anti-state activity and for breaking rules of law. The movement of hippies not only remains dangerous for mental and physical health of youth but it also breaks its ethical and social values (Chróścielewski 1973).

[3] The phenomenon of "git-men" is related to social, behavioural maladjustment of a clearly criminogenic character. The "gits" included mostly boys lacking the appropriately shaped sphere of higher emotions, with emotional deficiency. They are powerful only when they are in a group. Their thesis of the so called "another life", expressing dysfunctional character of educational forms in re-socialization institutions (informal leadership of "git-men", "grypsers"), a specific code of rules obligatory within the walls of punitive and corrective institutions was taken out of the institutions and it provides basis for strong reciprocal bonds within the informal group solidarity.

were preserved from his pre-war activity in scout organization (Figure 2). He was also one of the founders (together with professor Maria Chmielowa and professor Stefan Flieger) of the Society of Alumni of Poznań Medical School.

Let the reminiscences involving one of professor Chróścielewski's activities, his passions, help to extend memory of this greatly deserving person not only for forensic medicine but also for Wielkopolska region.

Figure 2. Professor Edmund Chróścielewski's drawing from his pre-war activity in scout organization

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