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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*.

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ORIGINAL PAPER

JMS Journal of Medical Science

Cervical expression of elafin and secretory leukocyte peptidase inhibitor does not predict preterm delivery in twin pregnancy – results from a pilot study

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ABSTRACT

Introduction. Elafin and secretory leukocyte peptidase inhibitor may serve as the predictors of cervical shortening and preterm delivery in twin gestation.
Material and Methods. A prospective observational study was conducted between September 2016 and March 2017. Cervicovaginal swabs collected from 40 women with twin gestation were analysed and the mRNA expression of elafin and secretory leukocyte peptidase inhibitor (SLPI) correlated with preterm delivery.
Results. The mean gestational age at delivery was 35.6 ± 5.8 weeks, with 23 women delivering before 37 weeks (57.5%), 7 before 34 weeks (17.5%) and 3 before 32 weeks of gestation (7.5%). The mRNA expression of elafin and SLPI was not dependent on chorionicity and did not correlate with gestational age at delivery.
Conclusions. Elafin and SLPI are not appropriate predictors of preterm delivery in twins.

Introduction

Elafin and secretory leukocyte peptidase inhibitor (SLPI) are the members of the antimicrobial peptide family, playing an important role in the modulation of the immune system. They are major protease inhibitors secreted at mucosal surfaces and the first line of defence against foreign antigens. They

are expressed throughout the female genital tract, inhibiting bacterial, viral and fungal activity and present anti-protease activity on proteases induced by infection and inflammation on the epithelial surfaces [1]. They belong to the whey acidic protein (WAP) family, as SLPI has two WAP domains and elafin has one WAP domain, which serve as neutrophil elastase inhibitors. Elafin, also known as peptidase inhibitor 3 (PI3), is a 9.9 kDa protein which is the C-terminal region of trappin-2. It inhibits neutrophil elastase and proteinase 3. Elafin expression was reported to be elevated in fetal membranes of preterm prelabour rupture of membranes (PPROM) [2] and the cervicovaginal fluid of women delivering preterm in singleton gestation [3]. The PI3 gene produces a complex protein (12.3 kDa) which is split intracellularly into the mature form (9.9 kDa) and secreted into the extracellular matrix and transformed into soluble elafin [3]. Its production is stimulated by lipopolysaccharide and inflammatory cytokines and decreased by oestradiol [3]. SLPI is an 11.9 kDa protein and an anti-inflammatory mediator [4]. Its N-terminal domain presents activity against both gram-positive and gram-negative organisms. The C-terminal domain is a strong inhibitor of neutrophil elastase, cathepsin G, trypsin, chymotrypsin, tryptase, and chymase. SLPI negatively regulates proinflammatory signalling mediated by nuclear factor-kappa B (NF-kB) [4].

According to Romero, preterm delivery is a common symptom of various processes and causes [5], with infection being the etiologic factor in 30% of preterm deliveries in singleton pregnancies [5]. During gestation, the uterine cervix and its mucus play an important role in the protection from pathogens present in the vagina. If the barrier is insufficient, infection and inflammation trigger cervical remodelling and shortening which leads to delivery. Macrophages and neutrophils infiltrate the cervix and the local production of proteases begins tissue remodelling and cervical opening. Preterm delivery affects two-thirds of all twin pregnancies and contributes to 50% of all neonatal twin deaths [6]. In the Preterm Prediction Study, 54.4% of all twins were born before 37 weeks, 32% before 35 weeks and 8.8% before completing 32 weeks of gestation [7]. It is a major problem associated with multiples, so finding an efficient predictor of preterm delivery in twin gestation is of great importance. It was hypothesised that elafin and SLPI may serve as the predictors of cervical shortening and preterm delivery in twin gestation.

Materials and Methods

For this pilot study of biomarkers in the prediction of preterm delivery in twins, a prospective observational study was conducted in the 1st Department of Obstetrics and Gynaecology, Medical University of Warsaw between September 2016 and March 2017. The study was approved by the Ethics Committee of the Medical University of Warsaw and was conducted according to the Declaration of Helsinki.

The inclusion criteria were a twin pregnancy beyond 22+0 weeks of gestation, chorionicity established and documented on the 1st trimester sonographic scan (two gestational sacs or the lambda sign for a dichorionic pregnancy; a single gestational sac or T sign for a monochorionic pregnancy), verified gestational age (GA), known GA at delivery, newborns birth weight (BW) and complete medical data on the pregnancy outcome and neonatal outcome. Pregnancies complicated by one or two foetal demises, genetic or major anatomical abnormalities, twin to twin transfusion syndrome (TTTS), twin anaemia-polycythaemia sequence (TAPS), twin reversed arterial perfusion syndrome (TRAP), as well as monochorionic monoamniotic ones, were excluded from the study. GA was calculated based on the first day of the last menstrual period or a transfer day in assisted reproductive technique procedures and verified by the crown-rump length (CRL) measured on the first trimester scan (if estimated due dates were inconsistent and the difference was over 5 days, the ultrasound measurement was of primary importance; in case of CRL discordance, the measurement from the larger twin was chosen). Body mass index (BMI) was defined as the body mass divided by the square of the body height. Preterm delivery was defined as the delivery occurring before completed 37 weeks. PPROM was defined as amniotic fluid leakage before 37 weeks of gestation, without spontaneous uterine contractions. Gestational hypertension (GH) and preeclampsia (PE) were diagnosed according to American College of Obstetricians and Gynaecologists recommendations [8], whereas gestational diabetes mellitus (GDM) was according to the Polish Society of Obstetricians and Gynaecologists recommendations [9].

Women with dichorionic pregnancies were routinely counselled once every 4 weeks and in monochorionic pregnancies once every 2 weeks, including an ultrasound scan. In cases with no pregnancy complications, both monochorionic diamniotic and dichorionic twins were delivered beyond 37 weeks of gestation according to the local policy. All the women were counselled between 20 and 24 weeks of gestation. A routine ultrasound scan assessing foetal biometry and anatomy was performed during that period as well as an ultrasound measurement of the length of the cervical canal. This was measured according to the Foetal Medicine Foundation recommendations — a transvaginal probe was placed in the anterior fornix of the vagina with an empty bladder and the linear distance between callipers placed at the internal and external cervical os was taken. Also, cervicovaginal swab samples were collected with a special kit between 20 and 24 gestational weeks.

The primary outcome of the study was delivery occurring before the completion of 37 weeks of gestation. Secondary outcomes included deliveries before 34 weeks of gestation.

The RNA was isolated with a PureLink™ RNA Micro Scale Kit (ThermoFisher Scientific, MA, USA) according to the manufacturer's protocol. The quality and concentration of RNA were assessed with a NanoDrop spectrophotometer and reverse transcription was performed with a High Capacity RNA-to-cDNA Kit according to the manufacturer's protocol (Applied Biosystems). Gene expression was analysed by relative quantitation (RQ) using a comparative CT assay. Explants stimulated with the proangiogenic cocktail for PE stimulation and cells from the control group no. 1 for C199 stimulation were used as calibrators. Real-time PCR was performed on an Abi Prism 7500 (Applied Biosystems) in 96-well optical plates, with each sample run in triplicate and supplied with an endogenous control (human GAPDH no. Hs02786624_g1). The TaqMan Expression Assays (Applied Biosystems) SLPI: Hs00268204_m1 and elafin (PI3): Hs00964384_g1 were used and all probes were stained with FAM. Reactions were run in a 20 µl volume with TaqMan Universal Master Mix (Applied Biosystems), appropriate primer set, MGB probe and 5 ng of cDNA template and universal thermal conditions were used, i.e. 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. Data analysis was performed with sequence detection software version 1.2 (Applied Biosystems, ThermoFisher Scientific, MA, USA).

Data are presented as the mean (± SD), median or percentage. The Mann-Whitney test and Fisher's exact test were used for statistical analysis and a P-value < 0.05 was considered significant. The sensitivity, specificity, positive predictive value, negative predictive value and positive and negative likelihood ratio with 95% confidence intervals were calculated to test the predictive value for preterm delivery (before 34 and 37 weeks of gestation). The data were analysed using Statistica version 13.1. Test performance was described for the prediction of delivery before 37- and 34-weeks using receiver operating characteristic (ROC) curves, sensitivity, specificity and predictive values. Areas under the ROC curve (AUC) were calculated and compared. Correlations between biomarkers and the cervical length were assessed by the Spearman rank correlation test.

Results

This pilot study involved 48 women. One case of an intrauterine foetal demise was diagnosed and one premature rupture of membranes occurred at 21 weeks of gestation; they were excluded from further analysis. Six patients were lost to follow-up, finally, 40 patients were deemed eligible for analysis.

The cervicovaginal swabs collected from 40 women with twin gestation were analysed and their basic characteristics are presented in **Table 1**. A cervical length below 25 mm between 20 and 24 weeks of gestation was diagnosed in three women and they were administered progesterone vaginally (200 mg per day). No vaginal pessary or cervical cerclage were administered.

The median GA at delivery was 36 weeks (interquartile range 35–37), with 23 women delivering before 37 weeks (57.5%), 7 before 34 weeks (17.5%) and 3 before 32 weeks of gestation (7.5%). All patients gave birth beyond 30 weeks. Preterm delivery was spontaneous due to PPROM or regular uterine contractions in 16 women (11 monochorionic diamniotic vs. 5 dichorionic pregnancies; p=0.7) and 82.5% of women had a caesarean delivery. The indications for caesarean section were malpresentation of the first foetus (6 monochorionic vs. 3 dichorionic twins), inter-

twin growth discordance (2 vs. 1 respectively), PE (1 vs. 0 respectively) and maternal indications (2 vs. 1 respectively).

SLPI and elafin mRNA were detected in the cervical epithelial cells in all women and mRNA expression was not dependent on chorionicity (**Table 1**). There was no correlation between GA at delivery and the mRNA expression of SLPI or elafin. A separate analysis for the subgroup of women with spontaneous preterm delivery was performed, showing no relationship with biomarker expression [delta Ct SLP median 4.3, interquartile range (IQR) 3–4.9 in spontaneous preterm delivery groups vs. 3.4, 2.7–3.6, p=0.2; delta Ct PI3 2.6, 1.8–3.5 vs. 3.0, 2.3–3.5, respectively, p=0.5]. Women delivering beyond 37 weeks had a significantly longer cervix assessed by ultrasound. No significant correlation between cervical length and biomarker expression was observed (delta Ct SLP: Spearman rank correlation coefficient -0.08, p=0.6; delta Ct PI3: Spearman rank correlation coefficient -0.07, p=0.7).

The cut-off points for PI3 and SLPI mRNA were designated based on the ROC curves and are presented in **Figures 1A–D**. The AUC for both elafin and SLPI was similar ranging from 0.53 to 0.602. SLPI and PI3 had moderate sensitivity with low specificity in predicting preterm delivery before 37 and 34 weeks of gestation. The ROC curve was used to establish the cut-off point for cervical length in the prediction of preterm delivery, with a cervical length of 39 mm having sensitivity 561 (95% CI 32.2–.81.3), specificity 86.4 (95% CI 56.8–97.2), positive likelihood ratio 3.94 (95% CI 1.3–12.2) and negative likelihood ratio 0.51 (95% CI 0.29–0.91). There were no significant differences in the expression of SLPI mRNA (median 4.2, IQR 3.1–5.1 vs. 3.3, 2.4–4.4 respectively; p=0.3) or expression of PI3 mRNA (3.1, 1.6–3.8 vs. 2.8, 2.5–3.2; p=0.9) between women who had cervical length below or above 39 mm. The sensitivity, specificity, positive and negative likelihood ratios for delivery before 34 and 37 weeks, as well as cervical length less than 39 mm, are shown in **Table 2**.

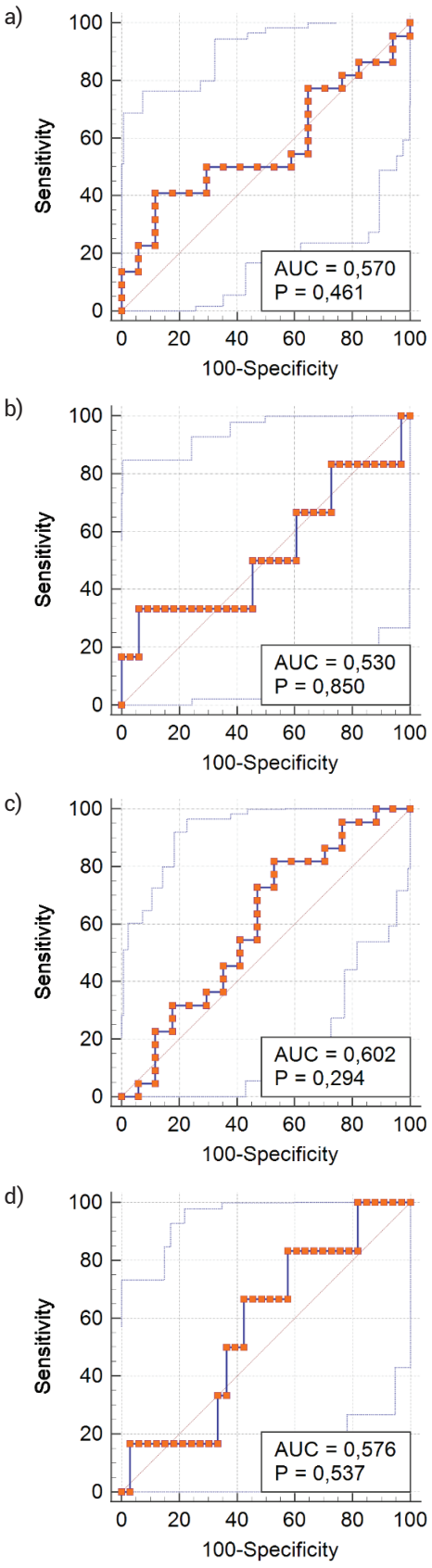

Figure 1. ROC curves demonstrating the ability of elafin and SLPI cervicovaginal fluid to predict delivery before 37 and 34 weeks of gestation in twins. a) elafin in delivery prediction <37 weeks; b) elafin in delivery prediction <34 weeks; c) SLPI in delivery prediction <37 weeks; d) SLPI in delivery prediction <34 weeks

Discussion

This is the first study of elafin and SLPI expression in the cervicovaginal fluid in twin gestation, demonstrating no significant differences in elafin and

Table 1. Characteristics of the study group

	Study group N=40		Hbd ≥37 N=17		Hbd <37 N=23		p	Hbd ≥34 N=33		Hbd <34 N=7		p
	N	%	N	%	N	%		N	%	N	%	
	median	IQR	median	IQR	median	IQR		median	IQR	median	IQR	
Age (years)*	33	31–35	32	28–34	33	31–36	0.6	32	30–34	33	32–36	0.5
BMI (kg/m^2)*	21.8	20–25	21.3	19.7–22.1	23	20.2–27.1	0.03	21.6	19.8–24.6	23.8	20.8–25.6	0.4
Gestational weight gain (kg)*	15	13–18	17	14.5–18	14.5	10–18	1	16	13–18	14	10–16	0.1
Primiparity	20	50	8	47.1	12	52.2	0.2	16	48.5	4	57.1	0.1
Chorionicity												
monochorionic	21	52.5	6	35.3	15	65.2	0.01	18	54.5	3	42.9	0.2
dichorionic	19	47.5	11	64.7	8	34.8	0.01	15	45.5	4	57.1	0.2
Nicotine addiction	4	10	1	5.9	3	13.0		3	9.1	1	14.3	
ART	9	22.5	4	23.5	5	21.7	0.8	7	21.2	2	28.6	0.4
DM	5	12.5	0	0	5	21.7	0.02	4	12.1	1	14.3	0.8
PIH	5	12.5	2	11.8	3	13.0	0.9	5	15.2	0	0	0.3
PE	1	2.5	0	0	1	4.3	0.8	1	3.0	0	0	0.9
GA at collecting cervical swab (week)*	21	20–22	21	20–22	21	20–22	1	21	20–22	21	20–22	0.9
delta Ct SLP*	3.9	2.9–5.1	3.4	2.6–4.6	4.4	3.5–5.1	0.3	3.8	2.9–5	4.5	2.9–5.1	0.5
delta Ct PI3*	2.96	1.8–3.7	3.0	2.3–3.7	2.9	1.4–3.6	0.5	3	2–3.7	2	1.1–3.5	0.8
Cervical length (mm)*	36	33–39	39	33–40	35	31–38	0.01	36	33–39	37	29–38	0.3
PPROM	10	25	1	5.9	9	39.1	0.01	7	21.2	3	42.9	0.01
Caesarean delivery	33	82.5	15	88.2	18	78.3	1	26	78.8	7	100	0.6
1st twin BW (g)*	2400	2080–2950	2750	2310–2990	2100	1620–2690	0.03	2540	1990–2940	1680	1390–1990	0.01
2nd twin BW (g)*	2280	1920–2790	2500	2010–2800	2050	1540–2580	0.04	2394	500	1660	1270–1910	0.01

BMI: body mass index, ART: assisted reproductive technology, DM: diabetes mellitus, PIH: pregnancy-induced hypertension, PE: preeclampsia, PPROM: preterm premature rupture of membranes

Table 2. Sensitivity, specificity, positive likelihood ratio and negative likelihood ratio of delta SLPI and delta PI3 for delivery < 34 and < 37 weeks of gestation

		Cut-off	Sensitivity (95% CI)	Specificity (95% CI)	Positive likelihood ratio (95% CI)	Negative likelihood ratio (95% CI)
Delivery <37 weeks	Delta SLPI	4.028	58.8 (32.9–81.6)	59 (36.4–79.3)	1.18 (0.6–2.3)	0.82 (0.5–1.6)
	Delta PI3	2.518	70.6 (44–89.7)	50 (28.2–71.8)	1.41 (0.8–2.4)	0.59 (0.3–1.4)
Delivery <34 weeks	Delta SLPI	4.456	60.6 (42.1–77.1)	50 (11.8–88.2)	1.21 (0.5–2.8)	0.79 (0.3–1.9)
	Delta PI3	2.871	54.6 (36.4–71.9)	50 (11.8–88.2)	1.09 (0.5–2.6)	0.91 (0.4–2.2)
Cervical length <39 mm	Delta SLPI	3.827	62.5 (43.7–78.9)	71.4 (29–96.3)	2.18 (0.6–7.3)	0.52 (0.3–1)
	Delta PI3	3.295	50 (31.9–68.1)	85.7 (42.1–99.6)	3.5 (0.5–22.2)	0.58 (0.3–0.9)

SLPI mRNA expression in the cervical fluid collected in mid-pregnancy between term and preterm deliveries. Elafin and SLPI had low specificity and moderate sensitivity in the prediction of delivery before 37 and 34 weeks, which is in line with other studies conducted in singleton pregnancies. Manning et al. detected no differences in elafin concentration in the cervicovaginal fluid of women delivering prior to or beyond 37 weeks in a study of 135 women with a history of preterm delivery (PTD) or cervical surgery [10]. Hezelgrave et al. observed 405 women with a singleton pregnancy at a high risk of PTD and 214 women at low risk of PTD, showing that elafin was not increased in high risk women who developed cervical shortening and delivered prematurely. The AUC for the prediction of delivery before 37 weeks was 0.52 (0.44–0.59) and before 34 weeks was 0.64 (0.58–0.71) [11]. Another observational study of 104 singleton high risk pregnancies by Bastek et al. reported that elafin measured in the cervicovaginal fluid in mid-gestation was not a predictor of PTD [12].

Conversely, studies have confirmed a correlation between elafin expression and PTD. Itaoka et al. compared the mRNA expression of elafin in cervical swabs collected at 29 weeks of gestation in women at low and high risk of PTD, finding that the cervical mRNA expression of elafin was significantly higher in high risk women delivering preterm compared with high risk women delivering at term and low risk controls [13]. Similarly, Abbott et al. analysed the cervicovaginal fluid samples collected between 13 and 30 weeks of gestation of 74 asymptomatic women with a singleton gestation, showing that women who developed a short cervix had elafin concentrations 2.71 times higher than those who did not (CI 1.94–3.79, p = 0.0005). Elafin concentrations were 3-fold higher than in the controls when cervical shortening was first detected. Elafin in the cervicovaginal fluid collected before 24 weeks of gestation was significantly higher in women who had a spontaneous PTD (OR 1.79; CI: 1.05–3.05, p = 0.034) and elafin measured between 14+0 – 14+6 weeks of pregnancy was predictive of subsequent development of a short cervix (AUC 1.00, p = 0.008) within 8 weeks [3].

Similar findings concerning SLPI were published by Itaoka et al, who reported that the mRNA expression of SLPI in the cervicovaginal fluid was significantly higher in high risk women who delivered preterm than in low risk women, as well as in high risk women who delivered at term [13]. Conversely, Samejima et al. found no differences in SLPI concentrations between women delivering before and beyond 37 weeks gestation. However, they reported a significant correlation between cervical mucus SLPI concentration and inflammatory cytokines, such as IL-6 and IL-8, which play an important role in preterm delivery [4].

In the present study, elafin and SLPI mRNA were detected in all collected samples. Itaoka et al. detected elafin and SLPI mRNA throughout the gestation and postpartum period, with the elafin concentration unchanged throughout pregnancy, while higher levels were detected after delivery [13]. In contrast, the expression of SLPI mRNA was the lowest in the first trimester, increasing in the second and third trimester [13]. Elafin and SLPI were also detected in the cervicovaginal secretion by other authors [14,15]. Differences in elafin and SLP production are probably controlled by hormones, especially progesterone. It was demonstrated that progesterone exposure increased SLPI but not elafin mRNA expression in a breast epithelial cell line [16], which may explain the differences in the observed expression of these antimicrobial peptides during pregnancy.

As uncomplicated twin pregnancies delivery was scheduled beyond 37 weeks in both dichorionic and monochorionic diamniotic gestation according to our local policy, it is assumed that the comparison of preterm deliveries between both groups is reliable. There was no correlation between elafin or SLPI and preterm delivery in twin gestation, which may be due to the small study group size, as this was a pilot study of potential biomarkers in the prediction of preterm delivery in twins. However, the aetiology of preterm delivery in twin gestation is complex and may differ from that in a singleton pregnancy. In twins, the overdistension of the uterus plays a major role and maybe a triggering factor for preterm delivery, even in the absence of infection and inflammation. The larger uterine cavity in the case of multiple pregnancies causes overdistension, which increases the expression of gap junctions in the myometrium and oxytocin receptors, as well as increased prostaglandin production [17]. As the aetiology of preterm delivery may be different from a singleton delivery, elafin and SLPI may not play a significant role in preterm delivery in multiple gestation.

The elafin and SLPI production were assessed in this study by examining the expression of their mRNA rather than protein, as neutrophil elastase can interfere with the secretion of SLPI by forming a positively charged molecular complex with SLPI [13], thereby influences the measurement of protein concentration and induce bias. Hence, we considered mRNA expression to be more independent and objectively reflects protein production.

In conclusion, elafin and SLPI expression in the cervicovaginal fluid is not related to the risk of preterm delivery and not appropriate for the prediction of preterm delivery in twins.

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Conflict of interest statement
The authors declare no conflict of interest.

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ORIGINAL PAPER

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The effects of submaximal exercise on a treadmill on the recovery of the stiffness index and reflection index in men with untreated hypertension

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ABSTRACT

Aim. Increased arterial stiffness is a risk factor for cardiovascular disease (CVD). Adult men with untreated hypertension are at risk of future CVD. The study aimed to compare the effects of a submaximal exercise on the recovery of the two descriptors of arterial pressure waveform, i.e. stiffness index (SI) and reflection index (RI), between men with untreated hypertension (HA) and healthy peers.

Material and Methods. The study included 70 Caucasian men with untreated primary HA and 30 normotensive men. Blood pressure, SI and RI were determined by photoplethysmography before and 6 minutes after a submaximal (up to 85% of age-predicted maximal heart rate) exercise on a treadmill.

Results. Baseline SI was higher in HA than control men (6.06 [0.66] vs. 6.61 [0.84] m/s; p=0.0019) and remained significantly increased during post-exercise recovery only in HA men (7.59 [1.6] vs. 6.18 [0.85] m/s; p<0.0001). Pre-exercise RI did not differ between HA and healthy men (50.74 [14.17] vs 48.9 [14.86]%). Six minutes after the exercise, RI higher in HA patients than in healthy men (45.26[15.33] vs 36.2 [13.18]%; p=0.0058).
Conclusions. Arterial stiffness is higher in men with HA both at rest and 6 minutes after exercise. Compared with healthy men, those with HA have more increased arterial tone and impaired vasodilation but only during the recovery. It suggests that untreated HA patients have abnormal mechanical properties of arterial pressure waveforms during the post-exercise recovery.

Introduction

A submaximal exercise treadmill test (ETT) is an effective and readily available method to assess the blood pressure response (BP) during exercise. It is also an important diagnostic tool in cardiovascular (CV) system evaluation [1,2]. Typically, exercise induces an increase in systolic blood pressure (SBP) and no change or a mild reduction in diastolic blood pressure (DBP), with a decline of both during post-exercise recovery in healthy people [3]. However, hypertension is accompanied by a higher increase in SBP and no drop or even a raise of DBP. Nevertheless, both SBP and DBP decrease during recovery in patients with HA, consequently, regular exercise is recommended as a treatment for HA treatment [4-6].

Arterial stiffness is a recognised risk factor for CV disease (CVD) and is influenced by age, hypertension, male sex, diabetes, obesity and smoking etc. [7-10]. Increased arterial stiffness is a marker of subclinical organ damage in patients with HA [11]. As such, the European Network for Non-invasive Investigation of Large Arteries advises that arterial stiffness is evaluated in large and medium-sized arteries to estimate CVD risk, especially in hypertensive patients without evidence of organ damage [12]. Furthermore, epidemiological studies have revealed the predictive value of arterial stiffness for fatal and non-fatal CV events in patients with hypertension [13], renal failure [14], type 2 diabetes [15] and in healthy people [16].

Arterial stiffness and other mechanical properties of arterial pressure waveforms, e.g. arterial distensibility, tone or vasodilation, are mainly assessed at rest, either in sitting or lying subjects. Physical exercise, for example, induces several hemodynamic and metabolic responses during ETT. Post-exercise recovery is a resting phase after ceasing physical effort and is accompanied by several physiological adaptations like reduction in myocardial oxygen demand, SBP or heart rate, removal of excessive heat or lactate produced by working muscles [17,18]. These post-exercise changes are believed to be beneficial, particularly in people with CV risk factors and disease. However, arterial mechanical properties have not been extensively studied in hypertensive patients in post-exercise recovery. For this reason, we aimed to compare the effects of submaximal ETT on the post-exercise recovery of two descriptors of the arterial waveforms, i.e., the Stiffness Index (SI) and the Reflection Index (RI) in hypertensive and healthy men. SI indirectly quantifies how stiff the arterial wall is, i.e. it increases with more rigid and thick arteries that become less distensible, whereas RI reflects the net peripheral arterial wall tone and effects of vasodilation of smaller arteries and arterioles [15].

Material and Methods

Study design and test subjects

The study population comprised 70 Caucasian men with untreated primary arterial hypertension and 30 healthy men aged 20 to 40 years. The preliminary diagnosis of HA was based on BP measurements according to the current European Society of Cardiology and European Society of Hypertension (ESH) Guidelines [11] and confirmed or excluded in all subjects by ambulatory blood pressure monitoring by an oscillometric method (TM-2430, A&D Medical, Japan) using the same recommendations.

The exclusion criteria were: (1) a history of symptoms suggesting secondary hypertension; (2) coronary heart disease, diabetes, cere-

brovascular disease, or thyroid disease, current or past renal disease; (3) medications that may significantly influence the analysed parameters, including aspirin, steroids, statins, non-steroidal anti-inflammatory drugs, or psychotic agents; (4) body mass index (BMI) equal to or greater than 40 kg/m2; (5) left ventricular hypertrophy, cardiac arrhythmias, or atrioventricular conduction disturbances in the resting 12-lead electrocardiography (ECG); (6) any abnormalities in the serum concentration of sodium, potassium, chloride, uric acid, or creatinine, and C-reactive protein (CRP); (7) fasting plasma glucose above 7.0 mmol/l or glycated haemoglobin (HbA1c) above 6.5%. All blood samples were analysed in the Central Laboratory of the Clinical Hospital of Transfiguration (Poznan, Poland) using standard laboratory methods.

The study protocol was reviewed and approved by an independent ethics committee (Bioethics Commission at Poznan University of Medical Sciences, approval no. 258/08, issued 6 March 2008). Informed written consent was obtained before the first study procedure.

Measurement of baseline characteristics

All individuals underwent anthropometric measurements (weight, height) and BMI was calculated using the formula: BMI = weight [kg]/ (height [m])^2. Biochemical parameters were assessed in fasting serum. All patients ate a standard breakfast and ECG exercise testing was performed two hours later (detailed below). None of the participants smoked before the test. BP measurements were performed following the ESH guidelines on HA [11]. The resting pulse pressure (PP) was calculated as the difference between the systolic and diastolic BP: PP = SBP - DBP.

Exercise test

The CardioTEST Alfa System B612 was used for the ECG exercise test with CardioTEST software and a Treadmill B612 model C with the 12 lead ECG module (Aspel, Poland). According to the Bruce protocol, the test was conducted and stopped when subjects achieved submaximal exercise (85% of their maximum estimated heart rate according to the formula 220 - age).

Exercise tolerance was estimated with the metabolic equivalents (METs) computed automatically by the applied software for the ETT. One MET is defined as the amount of oxygen consumed while sitting at rest and is equal to 3.5 ml O2 per kg body weight x min [19,20]. The following parameters were considered during the interpretation of this test: (1) SBP, DBP and PP, and HR at rest, at the peak of exercise and six minutes after the exercise during recovery.

Evaluation of mechanical properties of arterial pressure waveforms

Arterial stiffness was measured by digital volume pulse (DVP) photoplethysmography using a Pulse Trace PCA2 (Micro Medical Ltd, Rochester, UK). DVP was recorded in the subject's right index finger. The measurements were performed in triplicate (recorded for 30 s), an average was calculated and used for the analysis. All the patient measurements were performed on the same day by the same operator [21]. An explanation of how SI and RI were derived and computed from the DVP analysis is shown in Figure 1 [21]. SI reflects the stiffness and arterial distensibility [22], and the SI from DVP correlates highly with aortic PWV measured by other methods [23]. RI reflects the vascular tone of small vessels and estimates peripheral vasodilation [24]. Both resting and post-exercise measures SI and RI were performed three times in the supine position; the respective means were used for calculations. As DVP should be applied during the resting condition, neither SI nor RI was measured at the exercise peak like HR or BP parameters.

Statistical analysis

The Shapiro-Wilk test was used to evaluate the normality of the distribution of continuous data, which were presented as the mean and standard deviation (SD). Paired t-tests were conducted for comparisons between rest, submaximal exercise and post-exercise recovery for healthy men and HA patients. Independent t-tests were conducted to determine differences between the two groups for each phase, i.e. either rest or submaximal exercise or post-exercise recovery. Statistical analyses were performed using the MedCalc® Statistical Software version 19.5.3 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020) and two-sided tests, with the significance set at $p < 0.05$.

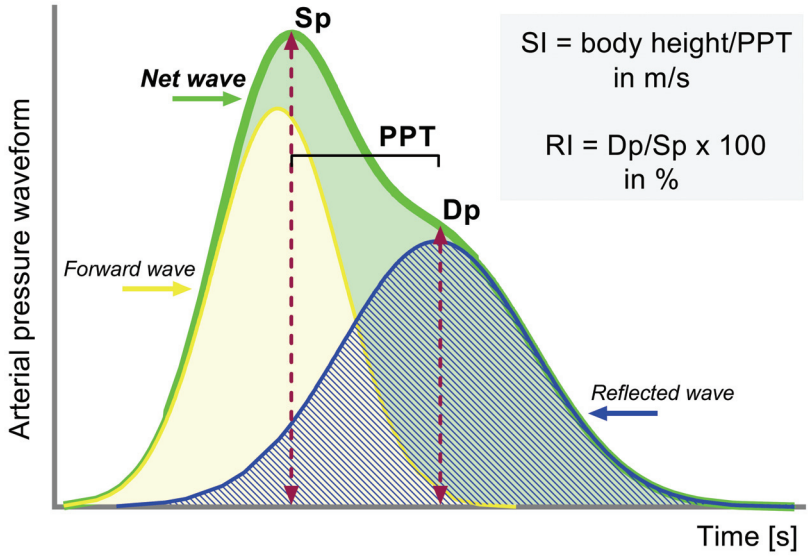

Figure 1. Schematic representation of arterial pressure waveform, its systolic peak (Sp), diastolic peak (Dp), the distance between the two peaks (peak-to-peak time – PPT). The Stiffness Index (SI) is obtained from subject height divided by the time between Sp and Dp, whereas the Reflection Index (RI) is determined as the ratio of the height of the Dp to the Dp [33]

Statistical analysis

The Shapiro–Wilk test was used to evaluate the normality of the distribution of continuous data. Their summary is presented as the mean and standard deviation (SD). The comparisons between rest, submaximal exercise and post-exercise recovery were made with the paired t-test for healthy men and HA patients. The Comparison between these two groups for the same phase, i.e. either rest or submaximal exercise or post-exercise recovery, was made in the t-test for independent (unpaired) data. Statistical analyses were performed using the Statistica 10 software (StatSoft Polska, Sp. z o.o., Krakow, Poland). The results were carried out using two-sided tests, and significance was set at $p < 0.05$.

Results

Baseline characteristics

Baseline clinical characteristics of all subjects are shown in **Table 1**. Men with HA were significantly older (app 3.3. years) but the mean age difference of 3 years does not seem to be of clinical relevance in our relatively young group. HA patients had a higher BMI of about 4.3 kg/m^2 and body weight of over 14 kg, and their achieved 2.5 less METs during submaximal exercise.

Table 1. Baseline clinical characteristics of healthy men and hypertensive subjects. Data are presented as mean and standard deviation (SD). Comparisons are made with the unpaired t-test between healthy and HA men BMI, body mass index: MET. metabolic equivalent

	Healthy men N=30		Hypertensive men N=70		
	Mean	SD	Mean	SD	P-value [a]
Age, years	24.37	2.43	28.13	5.27	0.0003
BMI, kg/m^2	23.21	2.16	27.56	3.77	<0.0001
MET	13.12	2.56	10.62	2.37	<0.0001
Waist, cm	82.13	6.63	93.71	11.40	<0.0001
Weight, kg	74.47	7.90	88.87	13.22	<0.0001
Height, m	1.79	0.07	1.79	0.06	0.8085

Abbreviations: BMI, body mass index; MET, metabolic equivalent

Table 2. Summary of paired (t-test) comparisons between rest, the peak of the submaximal exercise and post-exercise recovery for blood pressure, heart rate, SI and RI in healthy men and male patients with hypertension. Results are shown as mean and standard deviation Data are presented as mean and SD. Abbreviations: HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; SI, stiffness index; RI, reflection index; Rest-Smax, rest to submaximal exercise; Smax-6'Rec, submaximal exercise to 6 minutes post-exercise recovery; Rest-6'Rec, rest to 6 minutes post-exercise recovery

Parameter	Healthy men, N=30						Paired t-test			Hypertensive men, N=70						Paired t-test		
	Rest		Submax exercise		Recovery		P value – paired tests			Rest		Submax exercise		Recovery		P value		
	Mean	SD	Mean	SD	Mean	SD	Rest-Smax	Smax-6'Rec	Rest-6'Rec	Mean	SD	Mean	SD	Mean	SD	Rest-Smax	Smax-6'Rec	Rest-6'Rec
HR, 1/min	70.9	10.7	166.3	2.1	82.5	12.5	<0.0001	<0.0001	*<0.0001*	80.9	14.4	163.1	4.5	91.1	11.5	<0.0001	<0.0001	*<0.0001*
SBP, mmHg	120.1	8.4	158.2	14.4	122.8	8.6	<0.0001	<0.0001	*0.1083*	139.6	11.5	177.1	16.8	134.6	12.7	<0.0001	<0.0001	*0.0065*
DBP, mmHg	79.8	6.9	80.2	11.8	79.0	6.7	0.8160	0.6142	*0.6371*	88.3	8.7	93.7	11.5	84.3	8.9	<0.0001	<0.0001	*0.0002*
PP, mmHg	39.5	8.4	78.0	17.9	43.6	11.1	<0.0001	<0.0001	*0.0721*	50.9	13.2	83.5	19.8	49.9	12.6	<0.0001	<0.0001	*0.5729*
SI, m/s	6.1	0.7			6.2	0.9			0.4740	6.6	0.8			7.6	1.6			<0.0001
RI, %	48.9	14.9			36.2	13.2			<0.0001	50.7	14.2			45.3	15.3			0.0043

Data are presented as mean and SD. Abbreviations: HR, heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; SI, stiffness index; RI, reflection index; Rest-Smax, rest to submaximal exercise; Smax-6'Rec, submaximal exercise to 6 minutes post-exercise recovery; Rest-6'Rec, rest to 6 minutes post-exercise recovery

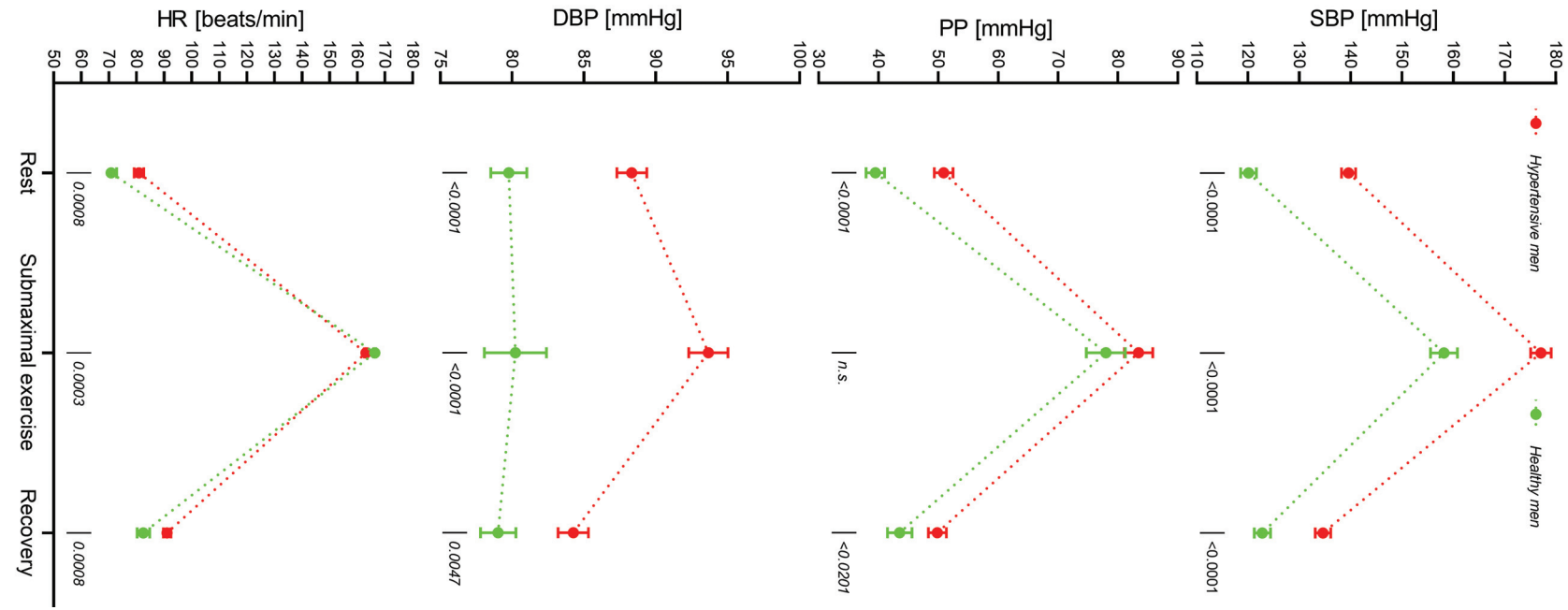

Figure 2. Comparison (unpaired t-test) of blood pressure and heart rate at rest, the peak of submaximal exercise and post-exercise recovery between men with and without hypertension. For details and abbreviations, please refer to the main text. Results are shown as the mean and standard error of the mean

Comparison of resting hemodynamic and arterial pressure waveform descriptors between healthy men and patients with hypertension

Figure 2 shows that the resting SBP, DBP, PP and HR were higher in HA patients than in healthy men.

The BP and HR during resting conditions, submaximal exercise and post-exercise recovery in healthy and hypertensive men are shown in **Table 2**. HR, PP and SBP significantly increased during submaximal exercise, then declined during recovery in both healthy men and those with hypertension. In HA men, DBP was higher at the peak of the submaximal exercise than at rest and declined during the recovery but was still higher than at rest. In contrast, neither submaximal exercise nor post-exercise recovery affected DBP in healthy men.

Compared with rest, SI was higher during post-exercise recovery only in HA patients, whereas RI declined both in healthy and hypertensive men (**Table 2**). HR, SBP, PP significantly increased in submaximal exercise and post-exercise recovery both in HA and healthy subjects. In contrast, DBP significantly declines in post-exercise recovery only in HA compared to healthy men. SI did not change in healthy subjects during post-exercise recovery, whereas SI was significantly higher in HA patients. RI significantly dropped in both groups during post-exercise recovery.

Resting and post-exercise SBP, DBP, PP and HR were higher in HA patients than in healthy men (**Table 2** and **Figure 2**). At the peak of submaximal exercise, SBP and DBP were higher while HR was lower in HA men. Also, SI but not RI was significantly higher in men with HA than in healthy subjects (**Table 2** and **Figure 3**).

Effects of the submaximal exercise and post-exercise recovery on hemodynamic and arterial pressure waveform descriptors in healthy men and patients with hypertension

HR, PP and SBP significantly increased during submaximal exercise, then declined during the

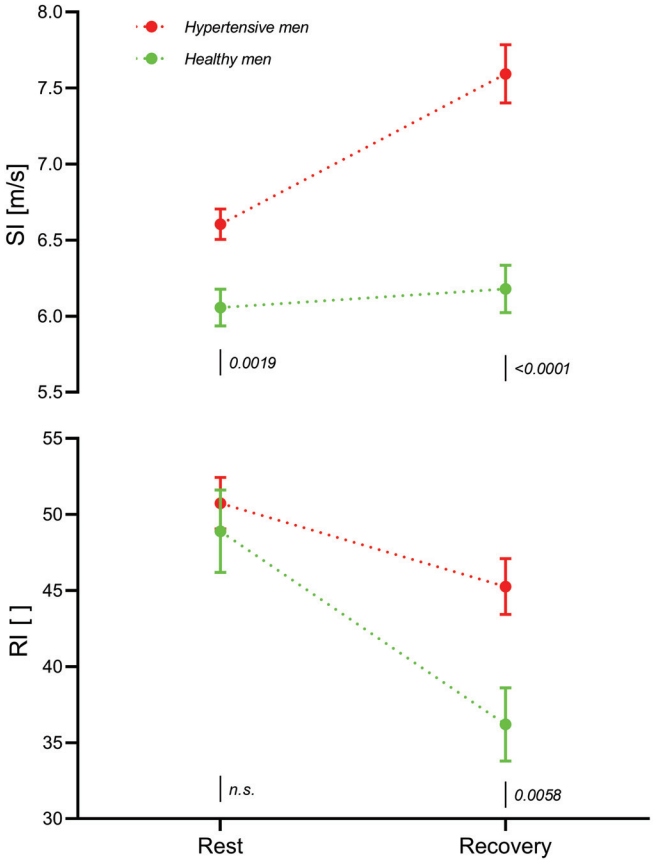

Figure 3. Comparisons (unpaired t-test) of the stiffness index and reflection index derived from the Digital Volume Pressure analysis of arterial pressure waveforms between healthy and HA men

post-exercise recovery both in healthy men and those with hypertension (**Table 2**, **Figures 2-3**). In HA men, DBP was higher at the peak of the submaximal exercise than at rest and declined during the recovery but was still higher than at rest. In contrast, neither submaximal exercise nor post-exercise recovery affected DBP in healthy men. Compared with rest, SI was higher during post-exercise recovery only in HA patients, whereas RI declined both in healthy and hypertensive men.

Comparison of hemodynamic and arterial pressure waveform descriptors between healthy men and patients with hypertension at the peak of submaximal exercise and 6 minutes of the post-exercise recovery

Figure 2 shows that SBP and DBP were higher at the peak of submaximal exercise and during recovery in HA men than healthy individuals. PP was significantly higher in HA patients only during the recovery. In contrast, HR was lower in HA men at the peak of the exercise, and then it was higher during the recovery in HA patients. **Figure 3** reveals that compared to healthy men, SI was higher and RI was lower in HA patients.

Discussion

In addition to significant BP and HR changes, we found that the arterial wall mechanical properties are modified by submaximal exercise both in HA and healthy men. After submaximal exercise, SI increased in HA men, with no change in healthy individuals. Although no difference in the resting RI was found between healthy subjects and HA patients, RI significantly decreased during post-exercise recovery in both groups. It is noteworthy that RI decline during post-exercise recovery was deeper in healthy than HA patients.

The results of the Baltimore Longitudinal Study of Aging [25] and the study by Kaess et al. demonstrated that higher arterial stiffness in normotensive individuals might predict hypertension development [26]. In our investigation, HA patients had higher baseline SI compared to healthy individuals of the same age, in line with the results reported by Millasseau et al. [10]. SI increased in HA patients but not in the normotensive group.

Regular physical activity is beneficial for the CV system, including BP and HR [4]. In older adults with multiple CV risk factors, short-term improvements in the arterial stiffness induced by a regular aerobic exercise were attenuated over the long term of no activity [27]. Nevertheless, in pre- and hypertensive subjects, arterial stiffness was not modified by aerobic training unless the exercise was associated with a substantial reduction in SBP and/or prolonged duration of the intervention [28].

To date, only a few studies have examined the impact of a single bout of physical activity on arterial stiffness. Sharman et al. studied the effects of a stable, low-intensity exercise at the level of 60% of the predicted maximal heart rate in healthy people of different ages as with and without hypercholesterolemia [29]. All participants exercised on an ergometric stationary bicycle for 10 minutes with the ergometer resistance adjusted to maintain a steady-state heart rate. The aortic pulse wave timing, the augmentation index and PP amplification at 2-minute intervals were measured during exercise and 10 minutes after completion. The exercise caused no significant differences in the aortic pulse wave timing, however, the augmentation index decreased and the PP amplification declined during the exercise regardless of the presence or absence of hypercholesterolemia or participant age. Hu et al. found that PP, which is related to the arterial stiffness and stroke volume, remained increased 3 minutes after an aerobic exercise on a cycle ergometer in healthy people [30]. Heffernan et al. found no statistical difference in the pulse wave velocity 15 and 30 minutes after exercise in young white and Afro-American healthy men regardless of race [31]. Tabara et al. observed no significant change in the augmentation index of healthy and sedentary elderly subjects who underwent mild-to-moderate aerobic exercise (dancing and hopping) for 30 minutes, followed by a 5-minute cooldown [32].

All the mentioned studies and our results show different effects of exercise on mechanical properties of arterial pressure waveforms during recovery. There are several reasons for this, such as the use of different exercise protocols, for instance, the exercise treadmill in our study, ergometric stationary bicycle in studies by Sharman et al. [29], Hu et al. [30] and Heffernan et al. [31]. Also, the intensity and duration of the exer-

cise were different, e.g. mild aerobic, submaximal or exhaustion, endurance or resistant with some lasting up to six minutes and others to thirty minutes. Moreover, the mechanical properties of arterial pressure waveforms were made with various methods (applanation tonometry, photoplethysmography) and parameters (pulse wave velocity, augmentation index, PP, PP augmentation, aortic pulse wave timing SI and RI). Additionally, the measurements were taken at different post-exercise recovery phases between 10 to 30 minutes or after the heart rate returned to the pre-exercise baseline level. Finally, various groups were investigated, healthy young or older people, hypertension, hypercholesterolemia or other risk factors, both genders or men only. Consequently, it is not surprising that there is no agreement on the effects of exercise on the arterial pressure waveform mechanical properties. Therefore, our findings cannot be easily compared with other studies on post-exercise recovery.

We show that baseline SI, directly associated with arterial stiffness, was higher in the HA than in healthy men, and it was still increased during the post-exercise recovery. In contrast, pre- and post-exercise SI remained unchanged in healthy men. Moreover, RI, related to the vascular tone of small arteries/arterioles and arterial vasodilation, remained reduced during the post-exercise recovery both in healthy people and HA men. Nevertheless, the degree of RI reduction during recovery appears to be attenuated in men with untreated primary HA. During the recovery, the decline of RI suggests that the tone of small arteries is reduced, possibly due to improved arterial vasodilation.

Exercise is a physiological challenge that triggers a series of hemodynamic changes. We demonstrate that some exercise effects on BP profile, HR and mechanical properties of arterial walls are still visible 6 minutes after the exercise is stopped. We compared the post-exercise effects only in healthy men and subjects with untreated HA. The employed model can be easily reproduced and applied to different clinical scenarios or to investigate the effects of various pharmacological agents, for instance, used in the treatment of HA, as well as research the long-term effects of regular endurance and resistant training on SI and RI in HA patients. According to the most recent ESC guidelines on sports cardiology and physical exercise, regular endurance training should last a minimum of 30 minutes a day at least five times a week and resistance training should be taken for a minimum of 15 minutes and be repeated at least three times a week in HA patients [4]. However, the consequences of such recommendations on the mechanical properties of arterial walls are unknown.

Some limitations of the present study must be acknowledged. First, the study was performed on men, thus the results cannot be extrapolated to women. Another limitation is that all studied participants were relatively young to make general conclusions for a wide age range. All patients with HA were untreated to avoid potential interactions of pharmacological agents on our results. However, as already mentioned, our model can be easily transferred to pharmacological studies in HA patients. Finally, we only applied indirect measures of arterial mechanical properties. Nonetheless, the photoplethysmographic DVP has been extensively used both in research and clinical studies in different groups of patients, including those with HA [33].

Conclusions

This study demonstrated that young and middle-aged hypertensive men exhibit higher baseline and post-exercise arterial stiffness parameters than similar age normotensive individuals. Furthermore, vascular tone declines but vasodilation improves during the post-exercise recovery, although the magnitude of these changes is attenuated in patients with HA. This model can be easily applied to study the effect of pharmacological antihypertensive therapy on the mechanical properties of arterial walls.

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Conflict of interest statement
The authors declare no conflict of interest.

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ORIGINAL PAPER

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Histological disorders of neurons of phylogenetically different parts of the cerebral cortex in partial, subtotal, stepwise subtotal, and total cerebral ischemia

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ABSTRACT

Aim. Measure of the histological changes in neurons in the parietal cortex and hippocampus of rats with partial, subtotal, stepwise subtotal, and total cerebral ischemia.

Material and Methods. Studies were performed on 84 rats. Partial cerebral ischemia was modelled by ligation of one common carotid artery. Subtotal cerebral ischemia was modelled by ligation of both common carotid arteries. Stepwise subtotal cerebral ischemia was performed by sequential ligation of both common carotid artery with 7-day, 3-day or 1-day intervals. Total cerebral ischemia (CI) was modelled by decapitation.

Results. When comparing the morphological changes of neurons in the parietal cortex and hippocampus, we observed that, with the aggravation of the severity of cerebral ischemia, there was a progressive increase in the number of hyperchromic shrivelled neurons and neurons with pericellular oedema. Modelling of more severe types of ischemic damage lead to pronounced morphological changes in neurons – a decrease in size, deformation of the perikaryon, and increase in the degree of neuronal chromatophilia with their wrinkling.

Conclusions. The smallest morphological changes in neurons were noted in the partial cerebral ischemia groups and subgroup 1 of stepwise subtotal cerebral ischemia, with an interval between common carotid artery dressings of 7 days. The most obvious morphological changes were observed in the conditions of total cerebral ischemia after 1 day. Changes in the parietal cortex and hippocampus were unidirectional, but in the parietal cortex, which is most sensitive to oxygen deficiency, they were more pronounced.

Introduction

The study of the brain in health and disease is an urgent and promising area of modern science and, in this regard, a frequent topic of dissertation research. Cerebrovascular and cardiovascular diseases are the most pressing medical and social problems in the world. Each year 450,000

people suffer a stroke, of which 75–80% are ischemic strokes. Cerebral ischemia leads to a number of general and local metabolic and functional disorders, the pathogenesis of which is complex, multifaceted, and largely unclear [1-5]. Adequate models of cerebral pathology may contribute to the detailed understanding of the pathogenesis of these disorders and, in addition, allow assessing the development of damage and adaptive mechanisms of the brain, which serves as a fundamental basis for improving the diagnosis, treatment, and prevention. In previous studies, histological disorders of neurons of phylogenetically different parts of the cerebral cortex were studied in partial, subtotal, total, and subtotal stepwise cerebral ischemia. However, it is important to analyse these disorders in a comparative aspect.

Aim

The aim of the study was to compare histological changes in neurons in the parietal cortex and hippocampus of rats with partial, subtotal, subtotal stepwise, and total cerebral ischemia.

Material and Methods

The experiments were performed on 84 male outbred white rats weighing 240 ± 20 g in compliance with the Directive of the European Parliament and Council No. 2010/63 / EU of 22.09.2010 on the protection of animals used for scientific purposes. The animals were kept in an air-conditioned room (22 °C) under mixed illumination on a standard vivarium ration with free access to food and water, and no more than five animals per cage. Protocols were reviewed and approved by the Ethical Committee of the Grodno State Medical University (protocol No 1, 14.04.2013). Studies were performed on animals represented by five groups of 6 rats each. Partial cerebral ischemia (PCI) was modelled by ligation of one common carotid artery (CCA). Subtotal cerebral ischemia (SCI) was modelled by ligation of both CCA. Graded subtotal cerebral ischemia (SSCI) was performed by sequential ligation of both CCA with 7-day (subgroup 1), 3-day (subgroup 2) or 1-day (subgroup 3) intervals. Lastly, total cerebral ischemia (TCI) was modelled by decapitation. Specimens were divided into two groups and kept at +16 ºC. The brain was harvested 1 hour and 1 day after ligation of the CCA in case of PCI or the second CCA in case of SCI and SSCI, or decapitation in case of TCI in each of the groups.

The control group consisted of sham-operated rats. The sampling of the material was carried out at a similar time after the sham operation. Changes in the size (area) and shape (form factor, elongation factor) of the neurons, as well as the degree of chromatophilia of their cytoplasm were compared with CI for 1 hour and 1 day. After decapitation, the brain was quickly removed and pieces of the cerebral cortex were fixed in Carnoy's fluid. Paraffin sections (5 μm thick) were prepared using a microtome (Leica RM 2125 RTS, Germany) and mounted on glass slides. The localisation of the parietal cortex and hippocampus of the cortex in the histological preparations of the brain was determined using a stereotaxic atlas [6]. Serial paraffin sections were stained with 0.1% toluidine blue according to the Nissl method.

Study of histological preparations, their microphotography, morphometry and densitometry of the chromogen sediment were conducted using an Axioscop 2 plus microscope (Zeiss, Germany), a digital video camera (LeicaDFC 320, Germany) and ImageWarp image analysis software (Bitflow, USA). In each animal, at least 30 neurons of the fifth layer of the parietal cortex and the pyramidal layer of the CA_1 field of the hippocampus were assessed, which provided a sufficient sample size for subsequent analysis. To assess the severity of ischemic damage to the cerebral cortex under CI conditions, changes in the size and shape of neuronal perikaryons, as well as the degree of colour of their cytoplasm (chromatophilia), were studied. The change in the size of neurons was assessed on the basis of their area, the shape of the perikaryons of neurons was assessed using the form factor ($4\pi S / P^2$ – the index of sphericity and folding), and the elongation factor – the index of sphericity (Dmax / Dmin) using the ImageWarp image analysis program (Bitflow, USA).

The area of neurons (S) and the elongation of their bodies indicated the state of the cytoskeleton and the water-electrolyte balance, which can be disturbed during cerebral ischemia. In the histological preparations, the number of pyramidal neurons per unit area of the cerebral cortex sections was determined.

Among the total number of neurons, cells were distinguished according to the intensity of the colour of the cytoplasm (chromatophilia): normochromic – moderately coloured; hyperchromic – dark; hyperchromic wrinkled – very dark, with deformed perikaryon; hypochromic – pale-coloured; shadow cells – unstained, with vacuolated nuclei; cells with pericellular oedema are shrunken, with enlightenment around the perikaryon. The number of each type of cells was counted.

A Kruskal-Wallis test with Bonferroni correction was applied to analyse the data (Statistica 10.0 software for Windows, StatSoft, Inc., USA). The results are presented in the form Me (LQ; UQ), where Me is the median, LQ is the boundary of the lower quartile; UQ is the boundary of the upper quartile. The differences between groups were considered significant at $p<0.05$.

Results

Compared with the control, there were no changes in the size and shape of the neurons perikaryon after 1 hour with PCI (**Figure 1**). However, in this group, both in the parietal cortex and hippocampus, there was a decrease of 18% ($p<0.05$) and 16% ($p<0.05$) in the number of normochromic neurons, as well as an increase of 65% ($p<0.05$) and 69% ($p<0.05$) in the number of hyperchromic neurons, respectively (**Figures 2–4**).

The absence of pronounced morphological changes in the simulation of PCI in rats was explained by the compensation of blood circulation along the circle of Willis. There were no differences in the severity of the changes noted in the parietal cortex and hippocampus. In animals with 1-hour SCI, the size of the neurons of the parietal cortex and hippocampus were 52%

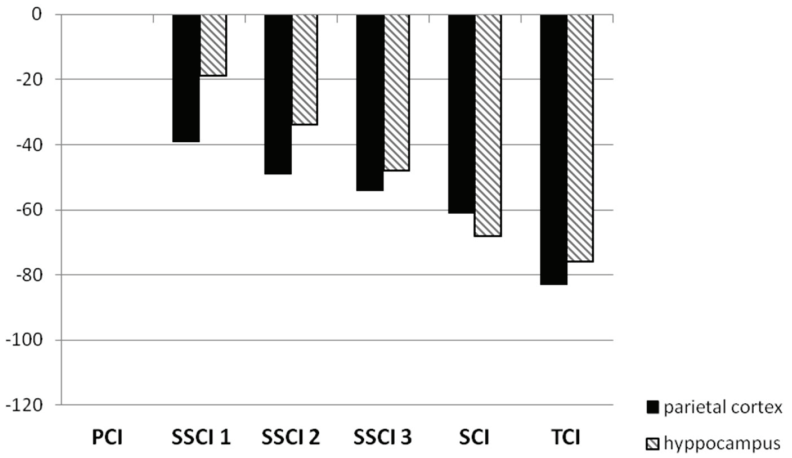

Figure 1. Changes in the area of perikaryon neurons in rats with cerebral ischemia (CI)

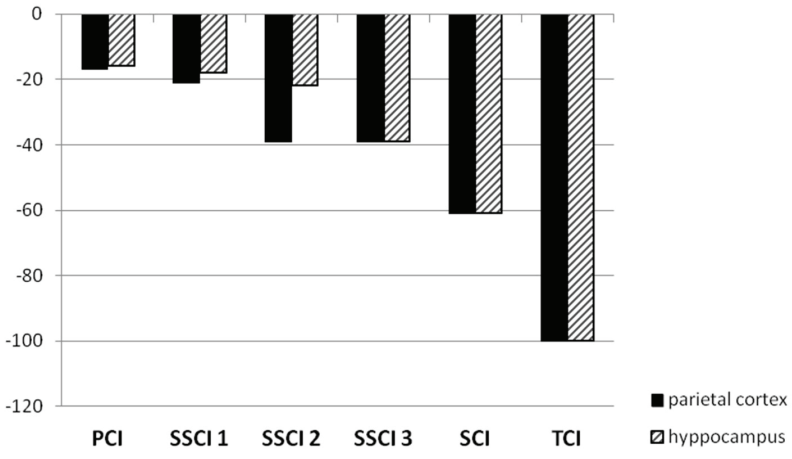

Figure 2. Changes in the number of normochromic neurons in rats with cerebral ischemia

(p<0.05) and 48% (p<0.05) smaller than in the control group, respectively. The elongation factor was 20% higher (p<0.05) in both studied regions, and the form factor decreased by 11% (p<0.05) and 22% (p<0.05) in the parietal cortex and hippocampus, respectively. Compared with the PCI group, the area of the neurons in rats with SCI decreased by 57% in the parietal cortex (p<0.05) and 42% in the hippocampus (p<0.05). At the same time, the elongation of neuronal perikaryons (elongation factor) increased by 20% (p<0.05) in each of the cortex sections, and their roundness (form factor) decreased by 11% in the parietal cortex (p<0.05) and by 22% in the hippocam-

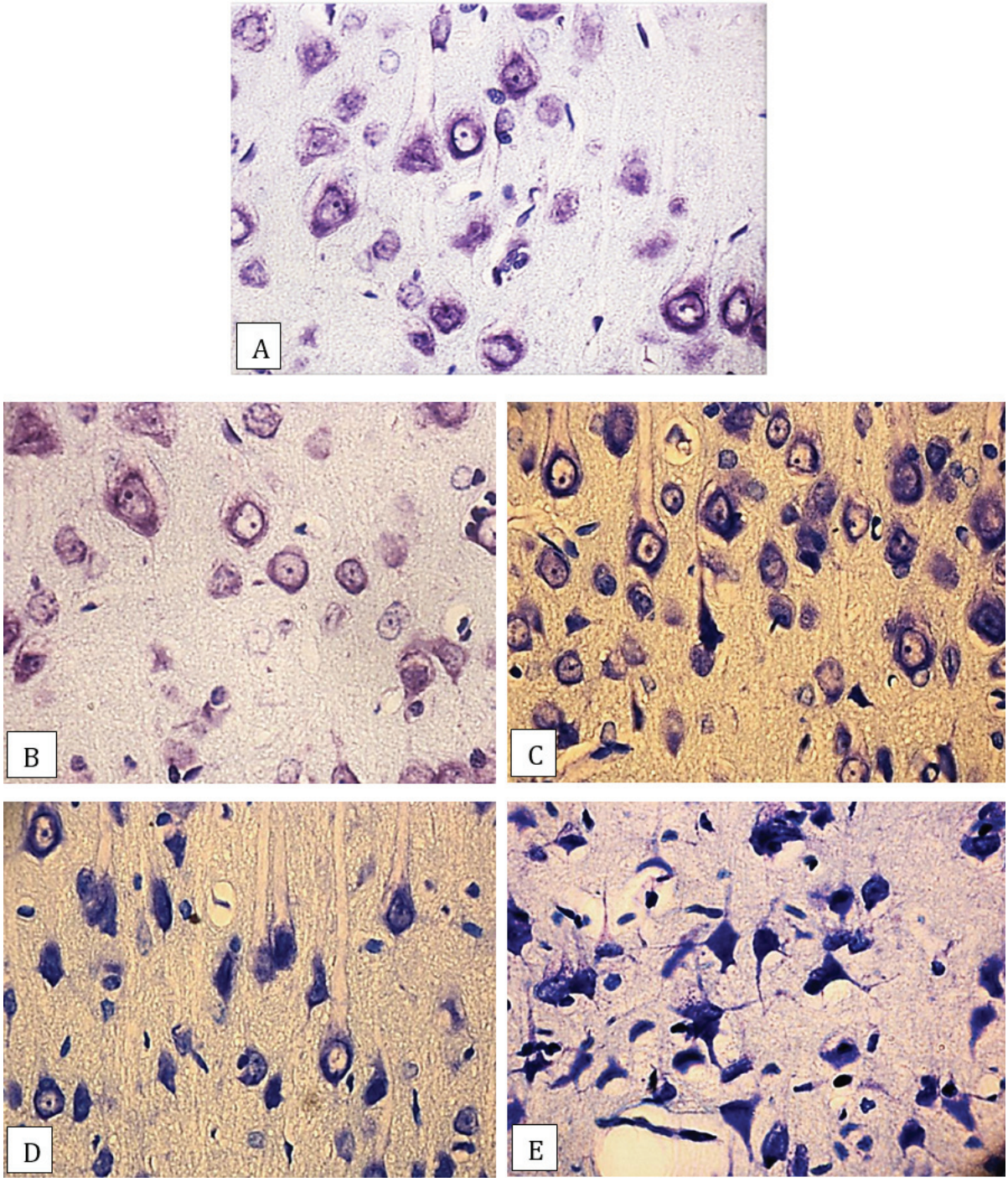

Figure 3. Neurons of the fifth layer of the parietal cortex in rats with a duration of the ischemic period of 1 hour. A – control, B – PCI (predominance of normochromic neurons), C – 1st subgroup of SSCI (predominance of hyperchromic neurons), D – SCI (predominance of hyperchromic and hyperchromic wrinkled neurons), E – TCI (absence of normochromic neurons, predominance of hyperchromic wrinkled neurons). Stained by Nissl. Digital microphotography. Scale bars and magnifications: A - 20 μm and x 400

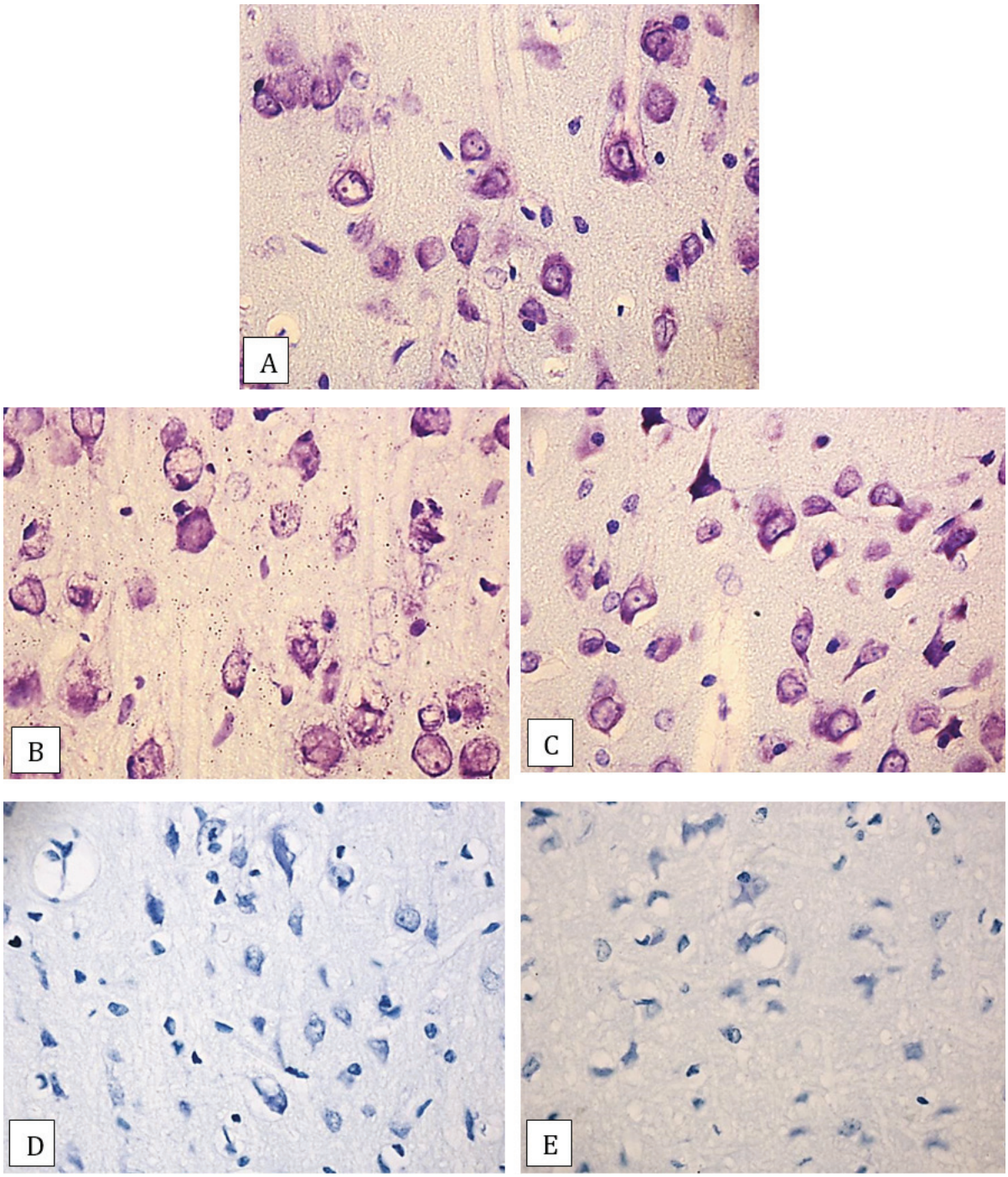

Figure 4. Neurons of the fifth layer of the parietal cortex in rats with a duration of the ischemic period of 1 day. A – control, B – PCI (predominance of normochromic neurons), C – 1st subgroup of SSCI (predominance of hyperchromic neurons), D – SCI (predominance of hyperchromic and hyperchromic wrinkled neurons), E – TCI (absence of normochromic neurons, predominance of hyperchromic wrinkled neurons). Stained by Nissl. Digital microphotography. Scale bars and magnifications: A - 20 μm. x 400

pus ($p<0.05$). When the ischemic period lasted 1 day, the disturbances were aggravated; compared with the control group, the area of the neurons decreased by 61% ($p<0.05$) in the parietal cortex and 68% ($p<0.05$) in the hippocampus. These changes were much more pronounced than in the group "PCI" lasting 1 day, where the area of neurons in the group "SCI" decreased by 53% in the parietal cortex ($p<0.05$) and 61% in the hippocampus ($p<0.05$). Compared with the 1-day PCI, the elongation factor with SCI increased by 33% ($p<0.05$) and 30% ($p<0.05$), respectively, and the form factor decreased by 33% as in the parietal cortex and hippocampus ($p<0.05$).

Compared with the control group, the number of hyperchromic neurons in the parietal cortex after 1 hour of SCI significantly increased by 79%, hyperchromic shriveled neurons by 80%, shadow cells by 60%, which is 39%, 85%, and 80% significantly more than in rats with PCI, respectively. In the hippocampus, the number of hyperchromic neurons increased by 77% ($p<0.05$), hyperchromic wrinkled cells by 95% ($p<0.05$), shadow cells by 100% ($p<0.05$), which is more than with PCI by 27% ($p<0.05$), 85% ($p<0.05$), and 67% ($p<0.05$), respectively. There were no differences in the number of pathological forms of neurons between the studied sections of the cortex. By day 1 of SCI, neurons with pericellular oedema 536 (536; 636.5) per mm^2 appeared in both studied structures. At the same time, their number in the parietal cortex was significantly higher than in the hippocampus by 25%. In rats with SCI, morphological changes were more pronounced than with PCI, but less than with SCI. The least pronounced changes in size and shape of the neurons were noted in the first subgroup (the interval between CCA dressings was 7 days). The area of the neurons, compared with the control, decreased in the parietal cortex by 39% ($p<0.05$), in the hippocampus by 28% ($p<0.05$), the elongation factor increased by 8% ($p<0.05$), and the form factor decreased by 11% ($p<0.05$). When comparing the "PCI" group and the first "SCI" subgroup, the shape of the perikaryon neurons in both studied sections was not significantly different. After 1 day, the sizes of the neurons in this subgroup were 36% larger ($p<0.05$) in the parietal cortex and 60% ($p<0.05$) in the hippocampus than with SCI, and compared with PCI, smaller by 27% ($p<0.05$) in the parietal cortex. In the hippocampus, the sizes of neuronal perikaryons did not differ from the sizes in the "PCI" group ($p>0.05$). In the second and third intervals (between CCA dressings was 3 days and 1 day, respectively), the decrease in the size and deformation of the neuronal perikaryons were significantly more pronounced than in the first subgroup. Compared with the control, the area of the neurons in the second subgroup decreased by 53% ($p<0.05$) in the parietal cortex and 40% in the hippocampus ($p<0.05$), the elongation factor increased by 14% ($p< 0.05$), and the form factor decreased by 22% ($p<0.05$) in both studied departments ($p<0.05$).

Compared with the "PCI" in the parietal cortex, the area of neurons decreased by 57% ($p<0.05$), and in the hippocampus by 31% ($p<0.05$), the form factor by 22% ($p<0,05$) and by 17% ($p<0.05$), respectively. The elongation factor increased significantly by 14% and 22% in the parietal cortex and hippocampus, respectively. Compared with the "SCI" group, no change was observed in the size of the neurons in the parietal cortex, but in the hippocampus, the area in the second subgroup "SSCI" was 15% larger ($p<0.05$). The form factor did not change, and the elongation factor significantly increased by 7% in rats with SCI both in the parietal cortex and hippocampus. Compared to the characteristics of the neurons in the control, the perikaryon neurons area in the third subgroup SSCI decreased by 60% ($p<0.05$) in the parietal cortex and 47% in the hippocampus ($p<0.05$). The form factor decreased by 11% ($p<0.05$), while the elongation factor increased by 20% in both studied regions ($p<0.05$). Compared with the group "PCI", the area of the neurons in the third subgroup with an ischemic period of 1 hour was 65% less ($p<0.05$) in the parietal cortex and 40% in the hippocampus ($p<0.05$).

The form factor did not change, and the elongation factor increased by 20% ($p<0.05$) in both studied departments. In the third subgroup, changes in the size and shape of neurons after 1 hour did not differ from those with SCI ($p>0.05$), but with an ischemic period of 1 day, the area of the neurons was 15% greater than with SCI, in the parietal cortex ($p<0.05$) and by 39% in the hippocampus ($p<0.05$). As the interval between CCA dressings decreased from 7 days in the first subgroup to 1 day in the third subgroup, more pronounced changes in the size (significant decrease), and shape of the neurons (a significant increase in the elongation factor and decrease in the form factor), and there was also an aggravation of changes in the chromatophilia of their cytoplasm. With SSCI, as the time interval between CCA dressings decreased, the number of normochromic neurons decreased ($p<0.05$) and the number of hyperchromic shrivelled neurons increased ($p<0.05$). The maximum number of hyperchromic shrivelled neurons was observed in the third subgroup (with an interval between CCA dressings of 1 day), especially in the parietal cortex (804 / mm^2; $p<0.05$), which is 42% more than in the first subgroup ($p<0.05$). In

addition, in the 3rd subgroup, one day after ligation of the second CCA, cells with peripheral oedema ($p<0.05$) appeared in the parietal cortex, which were already present in rats with SCI after 6 hours of ischemic period. After 1 day, their number in rats with SCI was higher in the parietal cortex by 53% ($p<0.05$). In rats with TCI, the most pronounced changes in size, shape, and degree of chromatophilia of the cytoplasm of neurons were observed.

With TCI lasting 1 hour, there was a significant decrease in the size of neurons by 74% and 50% in the parietal cortex and hippocampus, and with an ischemic period of 1 day, the area of neurons significantly decreased by 83% and 76%, respectively, which is more than with 1-hour TCI. Compared with 1-hour TCI, the area of neurons in the parietal cortex decreased by 34% and 42% in the hippocampus ($p<0.05$ for both). Aggravation in the shape of neurons was also noted. With an ischemic period of 1 hour, the elongation factor increased by 35% ($p<0.05$), while the form factor decreased by 34% ($p<0.05$) both in the parietal cortex and hippocampus. Comparing 1-hour ischemia with 24-hour TCI, the elongation factor increased by 25% in the parietal cortex ($p<0.05$) and 22% in the hippocampus ($p<0.05$). In animals with TCI lasting 1 hour, the majority of the cells in both studied sections of the cortex were hyperchromic wrinkled neurons (their number significantly increased by 96% compared to the control). Oedematous swollen neurons ($p<0.05$) appeared in rats after 1-hour TCI. The number of hyperchromic shrivelled neurons in the TCI group significantly increased by 80%, and the number of shadow cells significantly decreased by 30% than in the SIGM group. After 1 day of the ischemic period, cells with pericellular oedema began to predominate in the population of neurons ($p<0.05$). At the same time, normochromic neurons were absent ($p<0.05$), and single hyperchromic neurons were observed only with 1-hour of TCI ($p<0.05$). Compared with the "SCI" group, with 24-hour TCI, the number of hyperchromic shrivelled neurons and shadow cells decreased by 70% ($p<0.05$). A large proportion of the cell population consisted of neurons with pericellular oedema (2680 (2479; 3082) / mm^2), while in the SCI group such cells accounted for only 16% of the total number of neurons.

Discussion

In general, the smallest morphological changes in neurons were noted in the "PCI" groups and the first subgroup "SSCI", with an interval between CCA dressings of 7 days. With these CI modelling methods, adaptation processes occur that prevent the development of pronounced morphological changes and allow neurons to adapt to moderate hypoxia. For example, in PCI, the absence of pronounced morphological changes in rats is explained by the compensation of blood circulation along the circle of Willis. With SSCI, when the time interval is sufficient for the development of adaptive processes, the productivity of mitochondrial respiration increases [7,8], and possibly activating the production of nitrogen monoxide and hypoxia-induced factor. Modelling of more severe types of ischemic damage leads to pronounced morphological changes in neurons in the parietal cortex and hippocampus of the rat brain – a decrease in their size, deformation of the perikarya, an increase in the degree of neuronal chromatophilia with their simultaneous wrinkling and subsequent death. To the greatest extent, these violations were expressed in the third subgroup of SSCI with the shortest interval between dressings, which was 1 day and TCI. Hyperchromic neurons are regarded as ischemic-altered cells [9]. The appearance of shrivelled dark cells in hypoxic and anoxic conditions is a universal and most severe form of reactive and pathological changes in neurons, accompanied by changes in the metabolic rate, tinctorial properties of the cytoplasm, cell karyoplasm and varying degrees of ultrastructural changes in cytoplasmic organelles. Therefore, in hyperchromic wrinkled neurons, metabolic processes decrease, and the breakdown of nucleoproteins, especially nuclear ones, prevails over their synthesis. The reserves of ribonucleoprotein particles in the nucleus are preserved, but their excretion into the cytoplasm is blocked [10-14]. At later stages of ischemia, swelling of neurons is observed, accompanied by the dissolution of the chromatophilic substance, coarsening, disintegration and melting of neurofibrils, pycnosis of nuclei, and thickening and decay of processes. The neuropil is vacuolated and fragmented, undergoing granular-lumpy disintegration, and myelin dissolves, as a result of which lipid droplets begin to be detected along the nerve fibres [12-14].

Conclusion

When comparing the morphological changes of neurons in the parietal cortex with those in the hippocampus of the rat brain, it can be seen that, with the aggravation of the severity of cerebral ischemia, a progressive increase in the number of hyperchromic shrivelled neurons and neurons with pericellular oedema. The most obvious morphological changes (a decrease in the size and deformation of the perikaryons of neurons) were observed in the conditions of TCI after 1 day. Similar to TCI, but less gross violations, were found with daily SCI and in the "SSCI" subgroup with an interval between CCA dressings for 1 day. Changes in the parietal cortex and hippocampus were unidirectional, but in the parietal cortex, which is most sensitive to oxygen deficiency, they were more pronounced. The results provide a fundamental basis for further detailing the pathogenesis of ischemic brain damage and searching for an effective correction for this pathology.

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REVIEW PAPER

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Genotoxic impurities in pharmaceutical products – regulatory, toxicological and pharmaceutical considerations

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ABSTRACT

This article provides an overview of the most important aspects around the detecting and reporting of genotoxic impurities in the pharmaceutical industry. It focuses on relevant regulatory, toxicological, and pharmaceutical considerations. In this regard, the concept of Threshold of Toxicological Concern is explained and the most common genotoxic impurities are described. Furthermore, toxicological methods for genotoxic impurities screening are presented. Finally, the article emphasises several issues regarding further development.

Genotoxicity is defined as any detrimental modification of the genetic material irrespective of its causative mechanism, as per ICH guidelines ICH (S2) R1 Genotoxicity testing and data interpretation for pharmaceuticals intended for human use [1]. Screening for the genotoxicity of pharmaceuticals intended for human use is crucial with regards to safety during therapy, and it is warranted during non-clinical development by ICH M3 (R2) Non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals and ICH (S2) R1 guidelines [1,2]. Moreover, according to ICH M7 Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk guideline [3,4], the impurities present in a final drug formulation, including degradation products and reaction-related impurities (i.e. starting materials, reagents, intermediates, solvents, catalysts, etc.), also require genotoxicity profiling. Specifically, mutagenic impurities are DNA reactive substances with the potential to directly damage DNA even at low concentrations (equalling ppm level), causing mutations and thus potentially leading to neoplasia. The presence of unusually toxic (e.g. DNA reactive) impurities

has been of significant concern to the industry and regulators for a long time, mainly because lower thresholds (below 100-1000 ppm as mandated for conventional impurities) seem relevant in their case. Thus, analytical procedures applicable to active pharmaceutical ingredients (APIs) and drug products or the most commonly encountered impurities and degradants have been found to lack the appropriate level of sensitivity for adverse mutagenic effect detection [5,6]. Therefore, although sufficient as a framework for the qualification and control of the most commonly encountered impurities and degradants, ICH Q3A(R2) Impurities in new drug substances and ICH Q3B (R2) Impurities in New Drug Productscannot be applied to genotoxic impurities. Finally, ICH guideline M7(R1) was adopted in 2014 (current effective version dated Feb 2018) to supplement ICH Q3A, Q3B and M3 (R2) and to provide a practical testing approach to support the identification, characterisation, qualification, and control of mutagenic impurities in pharmaceuticals. Its purpose is to establish acceptable limits that guarantee negligible life-time risk of cancer [7].

The scope of application of ICH guideline M7(R1) excludes its retrospective use. In fact, it pertains only to new drug substances and new drug products which undergo the procedure of clinical development; there are several exceptions, including advanced cancer indications, biological/biotechnological, peptide, oligonucleotide, radiopharmaceutical or fermentation products, herbal products, and crude products of animal or plant origin. It also remains effective for post-approval submissions of marketed products, but only when there is new mutagenic data for the reported impurities or the introduced changes of synthesis and manufacture affect the impurity profile to the extent that the levels of existing impurities or degradants increase or unreported impurities or degradants appear [4]. Modifications of indication or dosing which significantly affect the acceptable cancer risk level will also result in the need to apply ICH M7 (R1). Importantly, the theoretical structural alerts alone will not trigger concern in marketed products unless the structure belongs to the group defined as the 'cohort of concern', which includes aflatoxin-like-, N-nitroso-, and alkyl-azoxy structures [4,7,8].

In new drug substances and new drug products, actual synthetic and degradation impurities exceeding the ICH Q3A/B reporting thresholds as well as the potential impurities which could be present in the final API or drug formulation must be evaluated for their mutagenicity. Potential impurities include starting materials, reagents, and intermediates in the route of synthesis [4,9]. As for degradation products, the requirement for mutagenicity assessment applies to all those observed above the ICH Q3A and ICH Q3B reporting thresholds during long-term storage. In addition to this, potential degradation products mandated for mutagenicity evaluation include all those observed above the ICH Q3A/B identification threshold during accelerated stability studies [9]. Initially, they must be screened for DNA-reactivity based on the literature research or an assessment of Structure-Activity Relationships, which predict bacterial reverse-mutation assay outcomes. This initial assessment is designed to establish whether individual impurities contain (or could be metabolised into) any electrophilic structural features that might constitute structural alerts for DNA-reactivity. Such compounds involve the following: alkyl esters of phosphoric or sulphonic acid, aromatic nitroso-groups, aromatic azo-groups, aromatic ring N-oxides, aromatic mono- and di-alkyl amino groups, alkyl hydrazines, alkyl aldehydes, N-methylo derivatives, monohaloalkanes, N and S mustards, propiolactones, propiosulfones, aromatic and aliphatic substituted primary alkyl halides, carbamates, alkyl N-nitrosoamines, aromatic amines and N-hydroxy derivatives, aliphatic epoxides and aromatic oxides, and aliphatic nitro group or halogenated methans [7,10-12].

One of most investigated genotoxic impurities are sulfonates. Sulfonate salts are frequently used in pharmaceutical optimisation due to their favourable physio-chemical properties, including a higher melting point, a limited tendency to form hydrates, and higher solubility [9]. For example, halperidol mesylate provides a higher dissolution rate which results in rapid onset of action. Sulfonic acid, however, can react with methanol, ethanol or isopropanol to produce sulfonate esters, which are recognised as potential alkylating agents. Methane sulfonate (MMS) and ethyl methane sulfonate (EMS) are well-established gentotoxic compounds in vitro and in vivo and, historically,

they have been reported as contamination in nelfinavir mesylate [13] and imatinib mesylate [14]. Moreover, alkyl halides, as electrophilic impurities, can be present in drug products secondary to the reaction of salt formation based on strong acid/base interactions in the presence of alcohol. Other electrophilic compounds commonly reported in drug products such as bethamethasone and atenolol are epoxides. Hydrazines, in turn, have DNA-forming adducts potential and are used as starting materials for pharmaceutical synthesis [9].

Nonetheless, at present the definition of the structural alert is not precisely settled. Thus, in some cases the correlation between the presumed structural alert existence and the real carcinogenicity might be weak [15]. For example, simple n-alkyl aldehydes, except formaldehyde, turn out to be negative in bacterial mutagenicity tests [16]. Similarly, out of acyl chlorides, acetyl chloride, chloracetyl chloride, and butyryl chloride are not mutagenic [7,17]. Furthermore, in the group of carbamates, the confirmed genotoxicity data only exists for vinyl carbamates, whereas the aromatic amines N-methylaniline and 4-aminophenol are not mutagenic [15]. This clearly indicates that the concept of structural alert applies only to selected compounds with that particular feature, giving rise to a certain proportion of false predictions. Therefore, the currently-adopted regulatory approach still demands a high level of expertise in chemistry and toxicology and it justifies a constant need for scientific endeavours to identify new mutagenicity data [15].

Based on the collected in-silico and literature data, the impurities are categorised into one of five classes. These classes, in turn, have relevant follow-up control actions: class 1 impurities are known mutagenic carcinogens; class 2 impurities are known mutagens with unknown carcinogenic potential; class 3 impurities demonstrate alerting structures (un-related to drug substance) with no supporting mutagenicity data; class 4 impurities show alerting structures (related to drug substance which is itself non-mutagenic); and class 5 impurities present no alerting structures. The impurity can be categorised as class 5 or 4 with no additional qualification studies if two complementary QSAR protocols (an expert rule-based and statistical-based) confirm no mutagenic concern or sufficient data is available to prove that there is no mutagenicity or carcinogenicity. If a structural alert is predicted or there is carcinogenicity scientific data available (Class 1 to 3 assigned impurities), Ames testing is required as a follow up action. If compounds exhibit a so-called structural alert, it is acceptable to maintain such an impurity at levels less than the Threshold of Toxicological Concern (TTC) (discussed later) without performing Ames testing on condition that the structural alert falls outside the cohort of concern (devoid of N-nitroso, aflatoxin-like, and alkyl-azoxy groups). The single bacterial reverse mutation assay (the Ames test) is the test of choice for mutagenicity and carcinogenicity prediction as it has exhibited a relevant sensitivity for the detection of genetic changes as well as for the majority of genotoxic rodent and human carcinogens [7]. This procedure has been adequately described in ICH S2(R1) and OECD 471 guidelines [1,18]. Specifically, the Ames test is designed to detect the point mutation-inducing capacity of the analysed substances. The procedure involves the exposition of amino-acid-dependent auxotroph Salmonella typhimurium or Escherichia coli strains to increasing concentrations of the tested impurity. Pre-existing point mutations in test bacteria render them incapable of growing and forming colonies in an amino-acid-deficient medium. The exposition to a mutagenic compound (tested substance) causes base substitutions or frameshifts within the mutated-bacterialgene and may cause a reversion to amino-acid prototrophy, thus restoring the revertant bacteria's ability to grow in the medium devoid of the amino acid essential for the parent test strain [18]. Some critical considerations regarding the Ames test as the sole reference for mutagenicity involve its positive/negative predictivity of approximately 80% and 50%, respectively. This indicates that a number of potential false positive and negative results may occur. There is also substantial interpretation uncertainty with regards to the Ames-negative and mammalian-cell genotoxicity assays-positive results [18].

For impurities classified as 1, 2, and 3, the acceptable human intakes are established. When the compound-specific risk assessment confirms carcinogenicity (class 1), the compound-specific acceptable limit for human exposure should be met. For class 2 and 3, where no carcinogenicity data is available, the conception of

the staged TTC can be adapted to each individual impurity [7]. TTC defines the permissible intake of a mutagenic impurity as 1.5 µg per person per day, which is deemed as bearing a negligible cancer risk for a long-term exposure exceeding 10 years [19]. Cancer risk, however, is assumed to increase as a function of cumulative dose, meaning that lifetime exposure to mutagenic impurity can be adjusted for a shorter duration of drug use according to its indications [7]. This concept would enable the higher daily intake of mutagenic impurities for the expected restricted treatment period, referred to as less-than-lifetime (LTL) exposure. In line with this, for a treatment duration of less than one month (for example, drugs used in emergency, antidotes, anaesthesia, acute ischemic stroke), 120 µg/day exposure to mutagenic impurity is acceptable. For treatment duration ranging from one to 12 months (e.g. anti-infective drugs) the allowed exposure is defined as 20 µg/day, and for treatments lasting 1-10 years (drugs used in diseases with shorter life expectancy) the exposure limit is reduced to 10 µg/day. The structural classes belonging to the 'cohort of concern', e.g. aflotoxin-like, N-nitroso or azoxy compounds are exempted from the TTC approach due to their high carcinogenicity potential. For several impurities commonly encountered in final drug formulations, the acceptable daily intake has been individually defined as demonstrated in **Table 1** [7].

Finally, for class 1-3 impurities there is a need for the development of control strategies to maintain their allowed limit. In addition, for the control of degradation products that are potentially mutagenic, a degradation pathway must be established and validated with regards to its relevance in real-time storage [7,9].

Although the current regulatory approach appears to exhibit a reasonable level of pragmatism, some issues still seem to lack clarity and precision. Unfortunately, the announcement of these rigorous requirements for genotoxicity screening did not prevent the N-nitrosodimethylamine (NDMA) crisis in 2018 and 2019, when the N-nitroso impurities were accidentally detected in ranitidine and valsartan-containing marketed products. This occurrence led to their global recall. It was further revealed that some of these drug products contained as much as 17 µg NDMA in a single tablet; the Food and Drug Administration estimated that this would lead to one additional case of cancer for every 8,000 patients taking the drug at the highest dose [20]. In March 2020, European Medicines Agency published a questions and answers document containing "Information on nitrosamines for marketing authorisation holders" to provide marketing authorisation holders (MAH) with guidance on performing testing for nitrosamine impurity. In line with this, MAHs are obliged to handle a risk evaluation to establish whether chemically synthesised APIs bear a risk of contamination with nitrosamines by 31 March 2021. In scenarios where risk is identified, these initial evaluations must be followed with a second step of confirmatory testing by 26 September 2022 for chemical APIs [21].

This event has ultimately proven that the scientific research for mutagenic impurities and degradation pathways of well-established pharmaceuticals remains absolutely essential.

Table 1. Acceptable daily intakes or permissible daily exposure of common drug impurities [7]

Impurity	µg/day
Acrylonitrile	6
Benzyl Chloride	41
Bis(chloromethyl)ether	0.004
1-Chloro-4-nitrobenzene	117
p-Cresidine	45
Dimethylcarbamoyl chloride	5
Ethyl chloride	1.810
Glycidol	4
Hydrazine	32
Methyl Chloride	1.361
Aniline	720
Hydrogen peroxide	68.000 or 0.5% whichever is lower
p-Chloroaniline	34
Dimethyl Sulfate	1.5

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Benefits of prenatal and postnatal vitamin D supplementation

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ABSTRACT

The best evidence for the role of vitamin D in infants is its influence on skeletal growth, however, the pleiotropic actions of vitamin D in the foetus and neonates are under-researched. The systematic reviews, based mostly on observational studies, suggest correlations between prenatal and postnatal supplementation and the occurrence of allergy, respiratory infections, sepsis or mental and behavioural development. Some of these studies focused on subgroups of neonates, such as preterm infants, investigating the influence inter alia on sepsis, bronchopulmonary dysplasia, and necrotising enterocolitis. Currently, there is a need for randomised trials for proof of the skeletal and pleiotropic effects of vitamin D in infants.

Introduction

The best evidence for the role of vitamin D (vit D) is its influence on skeletal growth [1]. Dietary or supplemental intake of vitamin D after intestinal absorption or from 7-dehydrocholesterol in the skin is bonded to vit D binding protein(DBP) in blood and converted to 25-hydroxyvitamin D (25(OH)D) in the liver, which is hydroxylated to the active form 1,25 dihydroxycholecalciferol in the kidney. The active form increases intestinal calcium absorption, renal calcium and phosphate reabsorption, regulating mineral homeostasis and parathormone secretion. Conversely, parathormone, calcium and phosphorus regulate the level of the active form. Severe vit D deficiency in children can lead to the development of nutritional rickets. In the first half of the 20th century, vit D supplementation during infancy became common, accompanied by a huge decrease in the incidence of nutritional rickets [2]. Conversely, the available data does not provide definitive evidence that vit D influences bone mineral density (BMD) in children [3]. Indeed, the data from

the most recent Cochrane database systematic review does not provide sufficient evidence to assess the influence of vit D deficiency in breastfed term infants on biochemical or radiological rickets and BMD. However, there is low-certainty evidence of maternal supplementation on the incidence of biochemical markers of rickets (14% decrease)in a subgroup of term infants with a high risk of vit D deficiency [4].

Preterm infants are particularly at risk of metabolic bone disease and vit D deficiency [5,6]. Additionally, vit D deficiency at birth due to lack of supplementation during pregnancy is a risk factor for reduced intrauterine bone growth due to inferior placentation [7]. The most recent meta-analysis of observational studies considered the influence of vit D deficiency (below 20 ng/ml) during pregnancy on the occurrence of small for gestational age infants and preterm birth [8]. The benefits of maintaining higher vit D levels in pregnant women to decrease preterm birth and small for gestational age infants were observed inter alia in studies with a large sample size conducted in California and Sweden [9,10]. However, other meta-analyses which also included randomised trials suggest that a deficiency rather than insufficiency is associated with preterm birth [11]. Low birth weight is associated with increased illness and mortality in infancy, consequently, reduced height in later life [12]. A lower areal BMD in early adulthood is more frequently observed in studies of preterm and very low birth weight infants [13,14] but it is not clear whether this can lead to osteoporosis in adulthood. Systematic reviews which evaluate the impact of vit D supplementation in pregnancy on infant BMD remain inconclusive [15], only confirming the prevention of neonatal hypocalcaemia in infants born to mothers who received vit D supplements [16]. A double-blind randomised trial with a good rate of follow-up conducted in Denmark demonstrated an association between high vit D supplementation during pregnancy (2400 IU) and 50% reduced odds of enamel defects in children at six years old [17].

Currently, the most prevalent role of non-skeletal vit D under investigation is its immunomodulatory impact. The vit D receptor is present on B cells, T cells, macrophages, and dendritic cells and can play a role in the immune response [18]. An inappropriate immune response influences the development of autoimmune disease. A systematic review based on observational studies suggests a correlation between early vit D supplementation in children and protection against the occurrence of type 1 diabetes, with dose response effects [19]. In a large sample size birth-cohort study conducted in Finland, children with high dose vit D supplementation (2000 IU) had a significantly reduced risk of type 1 diabetes [20]. The meta-analysis investigating inter alia the influence of prenatal and postnatal early vit D supplementation on allergic rhinitis, wheezing and asthma in children concluded that due to limited information, an early prevention impact still remains uncertain [21]. Nevertheless, a combined analysis of two large, randomised trials of vit D supplementation in pregnancy performed in the United States shows a significant 26% decrease in wheezing or asthma in the offspring by 3 years of age and was more pronounced for children whose mothers had achieved a sufficient 25(OH)D level [22]. However, in a Danish study, the protective effect of high vit D supplementation during pregnancy on persistent wheezing and the occurrence of asthma in the offspring at the age of 3 years was not observed at 6 years of age (limitation: target sample size was not achieved on follow-up). The Finish researchers performed the study based on the hypothesis of a positive correlation between vit D supplementation in infancy and an increased risk of atopy and allergic rhinitis in adult life [23]. Also, the potential vit D impact on food allergy among infants through promotion of immunological tolerance is under investigation. Since data from different observational studies are contradictory [24], there is a need to undertake a randomised trial targeted at this potential correlation.

Secondly, the vit D dependent immune response modulates the development of infectious disease. Vit D receptors can be found not only within the immune system but also on airway epithelial cells. Respiratory tract infections among neonates are mostly viral in origin, typically manifesting as wheezing, pneumonia or bronchiolitis. The influence of vit D supplementation in pregnancy and the neonatal period on the development and severity of the upper or lower respiratory tract infections in early childhood is currently under investigation in clinical trials. However, most studies concur that vit D status in young children modulates the occurrence and severity of respiratory infections [25,26]. In a

recent metanalysis, investigators examined the hypothesis that vit D can modulate the immune response in neonatal sepsis (NS) [27], an infection in the first 28 days of life including bloodstream infections, meningitis, and pneumonia. However, the analysis was based solely on observational studies and there was significant heterogeneity among the studies. Furthermore, an analysis was performed in a subgroup for an association between cord blood and maternal vit D level and the development of early-onset sepsis in term infants, showing a positive correlation between vit D deficiency during pregnancy and at birth and incidence of NS including early onset.

The influence of vit D on the immune response in preterm infants is a separate discussion. The most recent studies consider an association between maternal, cord or neonatal blood vit D level in a group of premature neonates and the occurrence of NS as well as necrotising enterocolitis (NEC), which in origin favour a pro-inflammatory mechanism [28,29]. In Cetinkaya's study, the maternal vit D level was a significant predictor of NEC, whereas in Say's study, the cord blood vit D level did not correlate with the risk of NS. Furthermore, antenatal vit D deficiency is linked to both airway inflammation and impaired anatomical and functional lung development which can lead to bronchopulmonary dysplasia in preterm infants. A meta-analysis based on four trials detected a significant association between vit D deficiency at birth and the development of bronchopulmonary dysplasia based on oxygen dependency at 28 days of age or 36 weeks of corrected age with no significant heterogeneity existing between the studies [30].

The last potential effect of vit D described in this short review is the effect on brain development, including neurotrophic and neuroprotective actions and changes in brain structure. A prospective study conducted on mother-child pairs reported a correlation between insufficient vit D pregnancy level and offspring language impairment at 5 and 10 years of age without offspring behavioural and emotional difficulties at any age [31]. Another large-scale prospective cohort study showed that higher 25(OH)D concentrations in pregnancy are associated with improved offspring mental and psychomotor scores in infancy [32]. The limitations of the study included the residual possibility of confounding by parental intelligence and lack of information on neonatal vit D supplementation. However, these results suggest that an optimal 25(OH)D level could improve early foetal brain development. Contrary to the latter research, a prospective cohort study with long term follow-up did not provide evidence inter alia that a higher maternal vit D status can support scholastic achievement among offspring [33].

Conclusions

The influence of vit D on neonatal health is one of the hot topics of the previous decade. The investigation of the skeletal and pleiotropic roles of vit D among infants is based on an increasing number of observational studies and their systematic reviews but there is a great need for randomised trials.

Abbreviations

Vit D - vitamin D; BMD - bone mineral density; NS - neonatal sepsis; 25(OH)D - 25-hydroxyvitamin D; NEC - necrotising enterocolitis

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Delirium in children – new research directions

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ABSTRACT

One of the problems associated with a patient's stay in the hospital and the procedures they undergo is delirium, the occurrence of which is associated with numerous complications, longer stay in the ward, higher risk of death and increased healthcare costs. Scientists are continuing to search for effective methods to minimise the risk of its occurrence, as well as to develop effective therapeutic procedures. One such area of the research is the identification of delirium biomarkers, which would allow further PK/PD (pharmacokinetic/pharmacodynamic) modelling. This article focuses on the emergence delirium, which occurs in patients recovering from general anaesthesia.

Definition and risk factors

Delirium is an acute and rapidly changing disorder of consciousness characterised by a decrease in the ability to focus, maintain or switch attention, accompanied by disorders of cognitive functions (memory deficit, disorientation, language disorders, hallucinations) and perception, which can be diagnosed based on medical history, clinical symptoms, physical examination or laboratory tests. According to the International Classification of Diseases ICD-10, delirium also includes emotional, psychomotor, and sleep-wake cycle disorders [1,2]. Delirium can occur in any setting, but is more common in a hospital setting, mainly in Intensive Care Units (ICU), due to associated clinical deterioration and/or pharmacological agents, such as benzodiazepines, opioids or anticholinergics, which may cause or exacerbate delirium [3-5]. Additionally, a hospital stay can

contribute to an increased risk of delirium due to exposure to stress, noise, excessive stimulation and the resulting disturbances of sleep and wake rhythm, diagnostic procedures, pain and fear related to a stay in the ward [6-12]. Injury, sepsis, any kind of shock, heart failure and respiratory failure have been associated with tissue hypoperfusion and development of inflammation, which may contribute to delirium [9,13].

Postoperative delirium (POD)

One form of delirium is postoperative delirium (POD), which can occur in patients who have undergone general anaesthesia for surgery, most often within three days of surgery. The development of POD is associated with a higher risk of death, a longer stay in the ICU and hospital, as well as cognitive impairment and the development of long-term psychiatric disorders, including symptoms of post-traumatic stress disorder [14-18].

Delirium in children

Children are particularly vulnerable to the occurrence of delirium, due to the dynamic development of their central nervous system, which may be disturbed under the influence of anaesthesia, and the consequences of this process may affect its further functioning. This is important because delirium can affect memory disorders in children, thus learning and intellectual development, even leading to sleep disorders, anxiety, and depression. Children's delirium significantly contributes to the increase in morbidity, mortality, and the costs of hospital care for sick children and adolescents. The current knowledge regarding the occurrence of this phenomenon in children is based mainly on extrapolation of the literature describing delirium in adults and on a few studies conducted in paediatric patients. Therefore, studies to assess the long-term impact of delirium on the development of children are necessary. Despite numerous evidence of a negative impact of delirium in paediatric patients, there are no standardised preventive, diagnostic and therapeutic measures [19]. Developing such rules could reduce the incidence of delirium among children, the length of their hospital stay and the associated costs; invasive interventions, such as prolonged mechanical ventilation or prolonged sedation, would be less necessary; ultimately improving the quality of life and the patient's condition [20].

Delirium in newborns and infants is especially difficult to diagnose due to communication limitations. In those age groups, symptoms of delirium include non-purposefulness, difficulty in engaging, agitation, restlessness and difficulty in calming the child [21-23]. Preschool children are most likely to develop delirium, which may be caused by their constant need for stimulation, the lack of which is due to immobilisation during the ICU stay and their increased sensitivity to disturbances in the sleep-wake rhythm [24]. Symptoms of delirium in schoolchildren and adolescents are easier to observe and are similar to those in adult patients [25].

Scales used to assess delirium in children include The Paediatric Anaesthesia Emergence Delirium (PAED) scale, which is used in children >2 yrs of age [26] and Cornell Assessment of Paediatric Delirium [27], a rapid observational screening tool to assess delirium in infants. The Cravero and Watcha scales are based on the assessment of levels of consciousness or arousal [28,29].

The importance of delirium prevention and current treatment

The importance of delirium for healthcare systems around the world is emphasised by LaHue et al. in relation to the COVID-19 pandemic caused by the SARS-CoV-2 coronavirus. The authors argue that in the face of a pandemic, there is a need to focus even more on preventing the occurrence of delirium, which is associated with an increased length of stay in the intensive care unit. They suggest that doubling the existing protocols for the prevention and management of delirium could significantly reduce the shortage of hospital beds and ventilators, essential in the fight against COVID-19 [30].

Prevention

To minimise the incidence of side effects of premedication drugs such as benzodiazepines, including the increased risk of delirium, non-

pharmacological methods of reducing preoperative anxiety are being sought. West et al. examined 59 children aged 3 to 10 years without coexisting anxiety disorders undergoing elective surgery. Some children were prepared for treatments according to standard procedures that included the use of a local anaesthetic prior to insertion of the needle, a brief consultation with an anesthesiologist and surgeon, and possible administration of anxiolytic drugs. For other children, in addition to the activities covered by standard procedures, "Child life preparation" was used, which consisted of role-playing with dolls and medical equipment, presenting children books and pictures showing activities related to the operating room as well as teaching them to cope with stress and finally translating planned activities according to the patient's age. The study showed a positive effect of additional preoperative activities in reducing the anxiety associated with the procedures by 13.8 points compared to the control group [31]. Another method of reducing preoperative stress in children was investigated by Dwairej et al., who used a combination of gradual accustoming children to the mask used to induce anaesthesia and distracting the child with video games, showing that such methods reduced anxiety during induction of anaesthesia compared to the control group [32].

Treatment

For the treatment of delirium, neuroleptic and antipsychotic drugs such as haloperidol, olanzapine, quetiapine or risperidone are used [33-36]. POD usually does not require the administration of drugs because it is short-lived, only non-pharmacological methods are used. Delirium in ICU patients lasts longer and very often requires medication, with haloperidol currently recognised as the gold standard delirium treatment. One of the other medicines used to prevent delirium in children is dexmedetomidine, a selective α2-adrenergic agonist in certain parts of the brain, which has a sympathetic, anaesthetic, anxiolytic, sedative and analgesic effect. Studies show that in ICU patients after surgery, dexmedetomidine reduces the need for midazolam, propofol and opioids both on an emergency basis and during sedation. The incidence of delirium measured on the CAM-ICU (Confusion Assessment Method for the ICU) scale after administration of dexmedetomidine was lower compared to midazolam. However, there is a need for further studies on the use of this drug, as there are no unambiguous data on the time of administration and optimal doses in the prevention of delirium [37].

Biomarkers

To evaluate the risk of developing postoperative delirium, the effectiveness of treatment and disease prognosis, it is necessary to select specific pharmacodynamic and disease progression biomarkers, which requires research and verifying which of them correlate well with the disease and treatment effectiveness. The most frequently mentioned in the context of delirium are: CRP (C-reactive protein), IL-6 (interleukin 6), IL-10, IL-8, PLR (platelet-to-lymphocyte ratio), PWR (platelet-to-white blood cell ratio), NLR (neutrophil-to-lymphocyte ratio), BUN/Cre (blood urea nitrogen / creatinine ratio), TNF-α (tumour necrosis factor α), as well as blood cortisol levels [37-49]. Some of the mentioned biomarkers, such as IL-6 and TNF-α, are also typical for the inflammatory response in the postoperative period, therefore it is necessary to specify the biomarkers most specific to delirium [50]. Genetic biomarkers such as the presence of the Apo-E4 alleles seem to be more exact [51].

Mathematical modelling

Dynamically developing mathematical modelling in medical sciences and population-based approach to data analysis is becoming more widely recommended for determining optimal pharmacotherapy protocols, especially in infants and children [52]. In July 2019, the FDA (Food and Drug Administration) published a new document on the significance and indications for population PK modelling in the pharmaceutical industry and clinical trials [53]. Modern mathematical modelling is not only used in the PK/PD drug analysis, but also in creating disease progression models and evaluating the influence of drugs on the course of disease [54].

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Rare diseases – a challenge for the medical world

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ABSTRACT

The diagnosis and treatment of rare diseases have improved significantly in recent years. The length of the diagnosis, which from the point of view of patients and their caregivers was considered the "Achilles' heel" of the healthcare system, has significantly shortened in many cases. Nevertheless, as research shows, there is still much to be done regarding the knowledge of rare diseases among healthcare professionals. The processes of diagnosis and treatment, as well as their organisation, should be redefined.

Rare diseases (RDs) have become a significant public health concern in recent years in many countries, with many previously ignored patients now becoming important. Many individuals and institutions are trying to support them in their everyday life, sometimes just to relieve their suffering. Public collections of money for expensive medical procedures often carried out in the best world centres have occurred. Moreover, such RDs are increasingly attracting media attraction, highlighting the importance of caring for such patients [1]. There is no single definition of RDs. In the European Union, a rare disease is one that affects no more than 1 person in 2,000 [2].

The aim of the study is to review the current literature on the subject, in particular the latest reports on the knowledge of students and healthcare professionals about RDs and the potential for changes in this area.

The public debate concerns both healthcare specialists participating in the diagnosis of RDs and diagnostic procedures used for patients with non-standard symptoms. Of course, the treatment process after the diagnosis of a specific disease is equally crucial, which raises the question concerning competence. The time required to reach a correct diagnosis is a key issue for RDs patients [3], with low awareness of RDs among physicians believed to be a significant reason for late diagnosis or misdiagnosis of RDs patients [4]. As one of the authors of a study noted, "[t]he lack of knowledge and experience with RDs,

combined with a limited ability to acknowledge this and act accordingly, is serious for the users and for the credibility of health professionals" [5]. The interest in the competence of medical personnel results, among others, from the "diagnostic odyssey" described by Black et al. [6], which encompasses three different periods: patient interval (starting from the first time the patient/caregiver notices what will later be classified as a symptom of the disease); primary care interval (beginning with the first patient visit to primary care); and specialist care interval, the time when the diagnosis is finally made. It seems that there are possibilities to shorten each of the three periods, however, unquestionably patients with RD currently face diagnostic delays. As McKay pointed out, "[t]he word 'odyssey' is not only used to highlight the average 5.6-year wait that people face before diagnosis, but it also conjures up an epic journey with giant-sized obstacles and detours along the way" [7].

In recent years, research on the knowledge and opinion of healthcare professionals and students about RDs has been conducted in several countries, e.g., in Belgium [4], Germany [8], Spain [9,10], and Poland [11-13]. The declared RDs-related knowledge of healthcare specialists and students does not seem promising for patients (**Table 1**).

The authors of the studies point to insufficient preparation, both of students and working healthcare professionals, to care for a patient with RD. It is recommended that the academic medical education on RDs be revised [4,12] and a continuous educational programme should be introduced [9].

Another crucial point identified in the research of individuals with RD as experts on their condition is that health professionals need to be conscious about the limits of their competence, and there is a need for revising the patient-provider relationship [5]. Research has shown that knowledge about RDs comes to a greater extent from the Internet than from university schooling, hence the existing medical education needs to be revised. Although the medical university curricula in various countries contain education on RDs, it is not standardised [7].

Assuming that the number of these diseases is estimated to be at least 7,000 [14], the question arises whether healthcare professionals can know about every RD and whether it makes sense to teach about so many uncommon diseases. As Van Groenendael et al. [15] pointed out, "[t]he current model of healthcare in Europe and beyond is well designed to cater for patients with common conditions. However, these care delivery models are not suited for patients with complex multisystem diseases with health needs that cross subspecialties", especially when conditions are rare. The frequency of misclassification of rare and non-rare diseases among medical students reflects the above-described problem and does not offer much hope for the proper diagnosis and treatment of RDs patients (**Table 2**).

Table 1. Declared healthcare specialists/students' knowledge of RDs depending on speciality and place of working [8,11,12]

Group	Declared knowledge (% of respondents)	
	Lack/Little	Good/Very good
Medical students	95.4	4.6
General dentists	77.7	22.3
Specialist dentist	81.9	19.1
Dentist – University employees	50.0	50.0
General nurses	97.4	2.6

Table 2. Misclassification of rare and non-rare diseases by medical students [11,13]

Rare disease misclassified as a non-rare disease	Respondents (%)	Non-rare disease misclassified as a rare disease	Respondents (%)
Multiple sclerosis	74 (13)	Munchausen syndrome	51 (11)
Cystic fibrosis	76 (11); 55 (13)	Crohn disease	34 (13)
Phenylketonuria	60 (11)	Fibromyalgia	33 (11)
Huntington disease	54 (13)	Halitosis	28 (11)
Sarcoidosis	52 (13)	Type I diabetes	5 (13)

Another problem is related to the RDs-dedicated healthcare system. It seems that the changes should include creating multidisciplinary units and high-quality practice guidelines [9,16] as well as digital platforms about RD symptoms [4]. Many specialists believe that RDs are so rare that the chance of encountering a patient with a specific RD is purely theoretical. However, with all RDs considered, the average doctor meets a patient with an RD on a daily basis [12].

Gaining experience in treating RDs is limited to specific diseases and specialised units of the healthcare system, therefore, progress in diagnosing, treating, and understanding a particular RD requires the synthesis of as much available data from multiple patients and institutions as possible [17]. It is worrying that for so many years, it has not been possible to create an effective clinical decision support system [18-20]. There is no doubt that such a system could support centres diagnosing patients who are likely to suffer from an undiagnosed RD. It is believed that general practitioners should also be an integral part of any initiative undertaken nationally to improve the diagnosis and management of RDs [21]. It seems that all healthcare professionals, as well as general practitioners, need formal training in RDs as a corner-stone of medical education [22,23], and perhaps patient-focused organisations could and should support such programmes [24].

Undoubtedly, a breakthrough in the diagnosis of RDs is exome sequencing. However, it should be remembered that currently in over 70% of patients in whom there was a high degree of pre-test suspicion for a monogenic RD, exome sequencing provides no molecular diagnosis [25]. Nonetheless, extensive and accurate phenotyping is essential to establish an appropriate link between potential candidate genes and disease characteristics [26]. In any case, it is the healthcare professionals, their knowledge and competence, that are responsible for the proper up-to-date diagnosis.

It is not known precisely how many patients suffer from RDs but certainly, there are more than anyone would have imagined a few dozen years ago, constituting a challenge for modern medicine. In order to address this challenge, two changes seem necessary. The first concerns the way of teaching at medical universities and the functioning of medical curricula. The second adjustment relates to the organization of the healthcare system with the implementation of new technologies supporting the diagnosis of RDs. The problem is not marginal and only well-trained staff and an adequately organised health care system can deal with it.

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Thousand words about alcohol use disorder in inflammatory bowel disease

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ABSTRACT

Patients with inflammatory bowel disease (IBD), especially those with severe disease and extraintestinal manifestations, are more frequently affected by anxiety and depressive disorders compared to the healthy population. This in turn may favour the expansion of alcohol use disorders but the role of alcohol consumption in the development of IBD and its impact on IBD course remains controversial. Importantly, ethanol is a significant factor contributing to liver failure and increased risk of various malignancies, including colorectal cancer (CRC). Primary sclerosing cholangitis (PSC) is a fatal extraintestinal manifestation of IBD leading to liver failure and promoting the development of cholangiocarcinoma and colorectal cancer. Indeed, alcohol abuse by patients with IBD and PSC may promote the progression of those complications but is difficult to diagnose. The underlying disease may cause similar abnormalities in laboratory and imaging tests to ethanol thus masking the problem, therefore gastroenterologists should pay special attention to the alcohol consumption of IBD patients.

Introduction

Inflammatory bowel disease (IBD) is a chronic gastrointestinal disease encompassing ulcerative colitis (UC) and Crohn's disease (CD). The complex pathogenesis of IBD remains unclear, however, according to the conception of the "IBDactome", diseases arise from the interplay between genetic, immunological, microbiological, and environmental factors [1]. IBD is characterised by a wide spectrum of extraintestinal manifestations (EMs), which may significantly impact the disease outcome. For instance, primary sclerosing cholangitis (PSC) is a fatal EM related to a higher risk of colorectal cancer (CRC) and cholangiocarcinoma. Furthermore, this chronic cholestatic liver disease may lead to liver failure, with more than 50% of individuals with PSC after 10–15 years of disease duration requiring liver transplantation (LTx) [2].

Case report

We report the case of a 26-year-old male patient with long-standing UC and PSC, hypothyroidism, depression, and nicotine addiction, repeatedly hospitalised in the Department of Gastroenterology and Hepatology due to disease flares or for monitoring of its course. The patient was admitted because of another endoscopic retrograde cholangiopancreatography to exchange the bile duct stents and start LTx qualification. During hospitalisation, laboratory tests showed signs of cholestasis (slightly elevated bilirubin, GGTP level over 50x above the upper limit of normal, alkaline phosphatase level close to 10x above the upper limit of normal), as well as elevated levels of aspartate and alanine aminotransferases. An extremely high level of thyrotropin was also alarming (over 20x exceeded the upper limit of the norm). The patient admitted the lack of regularity in the use of levothyroxine. Consequently, because of a previously diagnosed depressive disorder and in connection with qualification for LTx, a psychiatric consultation was performed that identified the harmful use of alcohol. The patient, despite previous numerous denials of alcohol consumption, admitted that he drinks mainly socially, consuming 6–8 beers a week with stronger drinks at the weekend despite knowing about the harmful effects of alcohol on the liver and is aware that he should stop. The psychiatrist recommended drug withdrawal psychotherapy for the patient. Further transplant qualification was postponed for the required abstinence period.

Discussion

The harmful use of alcohol is a drinking pattern that causes physical and mental damage, as well as psychological and social harm without being addicted to alcohol [3, 4]. According to the World Health Organization (WHO, 2016), Polish men annually consume 23.8 litres of pure alcohol and Polish women consume 8.3 litres, with approximately 12.8% of the Polish population, predominantly men, abusing alcohol [5]. The overwhelming majority of these people do not meet the criteria for addiction and only use alcohol in a harmful way. It is worth noting that more than 3 million people worldwide die from the harmful use of alcohol each year [6].

Alcohol has been frequently considered as a potential risk factor for UC because it can directly damage the intestinal mucosa, modify the gut microbiome, increase bacterial translocation and interfere with digestion and nutrient absorption [7]. However, in an EPIC cohort study in 2017 of 262,451 people from six countries, no association was found between long-term alcohol consumption and IBD risk [8]. In a meta-analysis of sixteen studies involving 3689 cases, of which nine studies assessed the relation between alcohol consumption and UC risk, there was no significant association between alcohol consumption and the risk of developing UC [9]. Nevertheless, some clinical studies suggest that alcohol consumption may exacerbate the disease in patients already diagnosed with UC, possibly due to the effects of alcohol on the immune system by increasing gut permeability and antigen exposure or to the high sugar content of alcoholic beverages and associated osmotic diarrhoea [10, 11]. Furthermore, an EPIC study demonstrated that alcohol consumption predisposes to the development of cancer including CRC. Some epidemiological studies suggest that even moderate drinking increases the risk of CRC, which is the third most frequently diagnosed cancer in both men and women globally. Alcohol not only causes toxic effects through carcinogenic metabolites such as acetaldehyde but alcoholics themselves are predisposed to a poor diet and disturbances of the circadian rhythm, which may further intensify carcinogenesis [12].

The risk of developing CRC may reach 30% 20 years after the diagnosis of concomitant IBD and PSC, therefore, alcohol abuse by patients with IBD and PSC significantly worsens the prognosis [13, 14]. However, a 2012 study conducted on 96 patients with PSC by the Karolinska Institute in Sweden showed that only a small percentage of PSC patients consume excessive amounts of alcohol, with patients with significant liver fibrosis reducing their alcohol consumption after PSC diagnosis [15]. However, it should be noted that despite a diagnosis of liver disease, patients may additionally abuse alcohol so the underlying disease will cause similar changes in laboratory and imaging tests as the consumption of ethanol, thus masking the patient's alcohol problem. The problem of alcohol abuse by PSC patients is of particular concern as most of them require liver transplants over time. An observational study

from France including 441 adult liver transplant recipients from 1991–2007 who survived > 6 months found that excessive drinking after LTx, regardless of the transplant reason, is associated with increased mortality [16]. Moreover, drinking alcohol after LTx is associated with an increased risk of graft rejection [17].

An additional argument for the active search for alcohol abuse is the fact that anxiety and depressive disorders are more common among IBD patients than in healthy individuals, and that they increase the risk of addiction to psychoactive substances including alcohol. In a study of 422 IBD patients at the Saarbrücken Clinic in Germany (2011), it was found that IBD patients with moderate/severe disease activity had higher rates of depression and anxiety compared to those with mild disease activity and age- and gender-matched healthy cohort. Additionally, the female gender was associated with an increased risk of anxiety in both IBD patients and the general population [18]. Inflammation, changes in the gut microbiota, and drug side effects influence the mental state of IBD patients but the relationship between mental disorders and IBD is not fully understood [19]. Many people with anxiety and depressive disorders tend to consume alcohol and other drugs, therefore it is important to pay special attention to drugs used by patients with IBD to provide them, if necessary, with timely psychological care to prevent addiction.

Summary

Patients suffering from UC and PSC are more predisposed to developing CRC than the general population, as well as to anxiety and depressive disorders, thereby the abuse of addictive substances. Alcohol can not only exacerbate IBD but more importantly, it also increases the risk of CRC, and like PSC, alcohol causes liver failure that often leads to LTx. Therefore, it is important that UC and PSC patients stop drinking alcohol and that gastroenterologists perform active screening for alcohol use disorders.

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Conflict of interest statement
The authors declare no conflict of interest.

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