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PROXIMAL BRACHIAL MONOMELIC AMYOTROPHY OR HIRAYAMA DISEASE: NO LONGER AN ALIAS? (case report)

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Background. *Brachial Monomelic Amyotrophy (BMMA) has been called as Hirayama disease (HD) when it is characterized by unilateral distal upper limb weakness and atrophy that shows progression for a limited period and is associated with typical features on MRI of cervical spine in flexion.*

Objective *was to explore the differences when BMMA affects the proximal upper limb muscles with the help of case report.*

Methods. *A case report of BMMA in an adult Indian male is represented.*

Results. *A 30-year-old man presented to us with a history of weakness in the proximal aspect of his left upper limb that began four years ago. The weakness was progressive up until 6 months prior to his presentation since when the weakness had neither worsened nor improved. Cervical spine contrast enhanced MRI revealed mild loss of cervical lordosis, but no features of HD like localized cord atrophy, loss of attachment of dura from subjacent lamina on neutral position axial T2WI MRI, nor any presence of posterior epidural crescentic enhancing mass on flexion contrast sagittal T1WI MRI. The patient was managed with supportive therapy and has been under regular follow up ever since. His clinical status has been stable.*

Conclusions. *We support the suggestion to consider proximal Brachial Monomelic Amyotrophy to be a separate entity and to be distinguished from Hirayama disease that should be reserved for patients with distal upper limb involvement with cervical MRI findings on flexion studies.*

KEY WORDS: **Brachial Monomelic Amyotrophy; Hirayama disease; cervical spine flexion MRI.**

Introduction

Traditionally Hirayama disease (HD), also known as Brachial Monomelic Amyotrophy (BMMA), has been described as a condition characterized by insidious onset unilateral upper limb weakness and wasting [1]. HD is considered to be a focal degenerative motor neuron disease (MND) affecting the cervical spinal cord. Though it is considered as a variant of motor neuron disease, it differs from other forms of MND, since it clinically involves only one limb and, after a brief period of progression, the neurological deficit becomes static [2].

Recently there have been arguments to recognize HD and BMMA as separate diseases with different clinical and neuroimaging features and therefore possibly different etiopathologies. Hassan K.M. et al. suggested that "Hirayama disease" should be used for cases with clinically unilateral upper limb distal muscle weakness and atrophy with cervical spine magnetic resonance imaging (MRI) demonstrating and the typical findings highlighted below, restricting the term "brachial monomelic amyotrophy" for cases of proximal upper limb atrophy without typical MRI features of HD [3].

Typical MRI findings in the cervical spine in Hirayama disease include [3]:

In Neutral position

- Abnormal cervical curvature (loss of cervical lordosis).
- Localized lower cervical cord atrophy with asymmetric cord flattening.
- Intramedullary hyperintensity in lower cervical cord.

Flexion position

- Loss of attachment between posterior dural sac and subjacent lamina.
- Anterior shifting of posterior cervical dural wall on flexion with Prominent epidural flow voids.
- Enhancing epidural mass in lower cervical region.

So, we report a case with isolated weakness and atrophy of proximal left upper limb muscles, without the typical MRI findings of HD.

Case study

A 30-year-old man, who was a farmer by occupation, presented to us in July 2015, with

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history of weakness in the proximal aspect of his left upper limb that began four years ago. The weakness was progressive up until 6 months prior to his presentation since when the weakness had neither worsened nor improved. There was no trauma or fever preceding the onset of weakness, no family history of similar complaints nor any exposure to toxins or heavy metals. He denied pain, numbness, diplopia, dysphagia, ptosis, muscle cramps, fasciculations, and headache or neck pain. Examination revealed marked atrophy and weakness in the proximal aspect of his left upper limb (Fig. 1).

Fasciculations were noted in the left deltoid. The left biceps and supinator reflexes were diminished. The rest of the motor system examination was unremarkable. Sensory and cranial nerve examinations were normal. Electroneuromyography (ENMG) showed denervation pattern with reduced motor action potentials and fasciculations in the left deltoid (Fig. 2).

Motor nerve conduction study showed reduced Compound Motor Action Potentials (CMAP) amplitudes in left axillary, musculocutaneous and infraspinatus, but the sensory nerve conduction was normal. Cervical spine contrast enhanced MRI revealed mild loss of cervical lordosis, but no features of HD like localized cord atrophy, loss of attachment of dura from subjacent lamina on neutral position axial T2WI MRI, nor any presence of posterior epidural crescentic enhancing mass on flexion contrast sagittal T1WI MRI (Fig. 3). The patient was managed with supportive therapy and has been under regular follow up ever since. His clinical status has been stable.

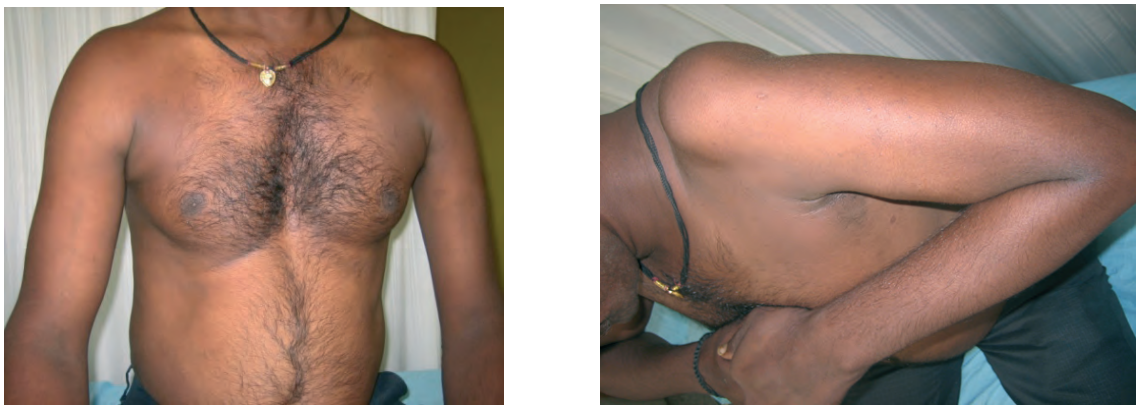

Fig. 1. Photographs displaying atrophy of the proximal aspect of the left upper limb with loss of contour of the left shoulder.

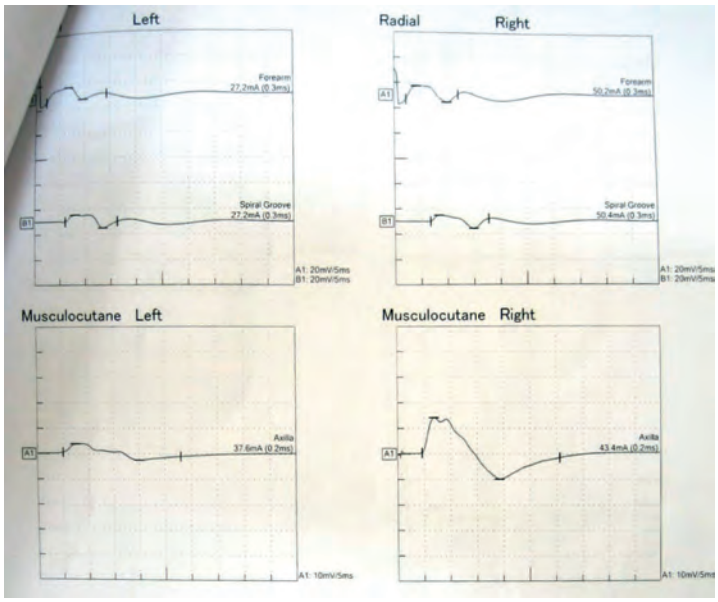

Fig. 2. Electroneurogram comparison of the nerves supplying upper limb muscles revealed reduced Compound Motor Action Potential (CMAP) on the left musculocutaneous nerve.

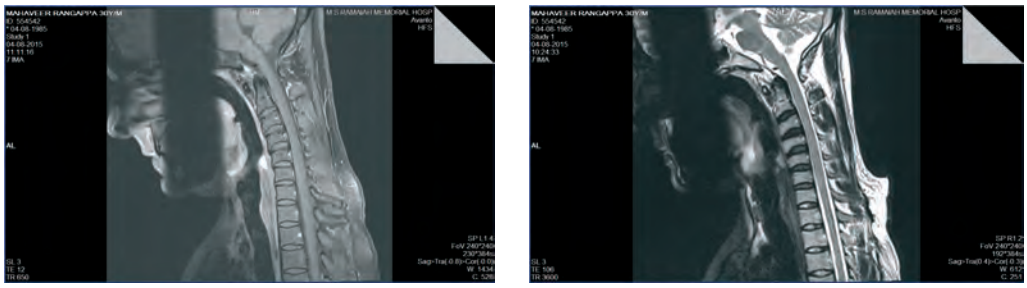

Fig. 3. MRI studies of the cervical spine in flexion failed to demonstrate the typical changes associated with Hirayama disease.

Discussion

Hirayama disease is a sporadic disease characterized by wasting and weakness confined to only one upper limb, initially progressing for 2-4 years which is followed by a stationary course, without clinical involvement of other limbs. There is no cranial nerve, cerebral, pyramidal tract or sensory involvement. In upper limb involvement, atrophy of the medial forearm muscles and the small muscles of hand are common [4]. EMG is characterized by fibrillations, positive sharp waves, and fasciculations consistent with the underlying pathophysiologic process of denervation [5]. MRI abnormalities in HD have been consistently reported in studies by Hassan et al., Sonwalkar et al. and Raval et al. [3, 6, 7].

The debate continues whether HD represents a focal primary lower MND or a local consequence of anatomical variation in the cervical spine [8]. It has been suggested that an imbalanced growth causes disproportional length between the patient's vertebral column and spinal canal contents, leading to tightening of dural sac and anterior displacement of posterior dural wall on neck flexion [9], thereby compressing the cord against the posterior margin of adjacent vertebrae producing microcirculatory disturbances in the lower cervical cord resulting in necrosis of anterior horn cells, gliosis and localized lower cervical cord atrophy, consistent with pathologic findings on autopsy [10].

As with any case presenting with unilateral upper limb weakness and atrophy, there are other differential diagnoses to be considered for this patient [11, 12]:

Multifocal motor neuropathy with conduction blocks involves upper limbs in asymmetrical manner with predominant distal muscle involvement, atrophy isn't a prominent feature and ENMG shows demyelination with conduction blocks and has high serum titers of anti-GM1 ganglioside antibodies.

In Spinal Muscular Atrophy and motor neuropathy, such as that due to lead toxicity, upper limbs involvement is bilateral and symmetric.

A chronic focal myositis can be differentiated by an elevated serum creatine phosphokinase, and the EMG, and the muscle histologic features.

Neuralgic amyotrophy or brachial neuritis is present with characteristic deep shoulder aching dysesthesia. There is good prognosis for full recovery.

An entity called "late-progression of poliomyelitis" merits a comment. In this disease there are: 1) a definite history of poliomyelitis; 2) residual but stable neurologic deficits; 3) a quiescent period of ten years or more. Our patient did not have any history of poliomyelitis in his past.

Cases of proximal brachial MMA reported in the literature are very limited, and no such cases have been shown to have dynamic compression of cervical cord on MRI. In 2000, De Freitas M.R.G. et al. published a case series of brachial MMA patients including one patient with proximal disease, in whom cervical MRI was normal [11]. In 2008, Orsini M. et al. reported two young men with proximal right upper limb MMA, and both had a normal cervical MRI [12]. In 2013, Hassan K.M. et al. also described two young males with proximal left upper limb MMA with only mild loss of cervical lordosis on MRI [3]. In 2015, Yoo S.D. et al. reported a patient with proximal upper limb atrophy with flexion cervical MRI showing typical features of HD, but this patient had certain atypical features such as left deltoid tenderness and upper motor neuron signs in ipsilateral upper and lower limbs [13]. In 2017, Aundhakar S.C. et al. reported a case of right upper limb wasting and weakness with typical MRI findings of HD but were involved of proximal as well as distal upper limb muscles [14].

Hence, it appears that proximal brachial BMMA may thus have a pathogenesis different from the dynamic ischemia of lower cervical cord postulated for distal upper limb wasting of HD. Perhaps such patients need not be subjected to cervical spine flexion contrast MRI; an MRI of the cervical spine in neutral position may suffice to exclude compressive myelopathy due to other causes.

Conclusions

We support the suggestion of Hassan et al. to consider proximal Brachial Monomelic Amyotrophy to be a separate entity and to be distinguished from Hirayama disease that should be reserved for patients with distal upper limb involvement with cervical MRI findings on flexion studies. Thus, there is a need to explore a different pathological mechanism for Brachial Monomelic Amyotrophy. We also suggest that performing flexion contrast MRI of the cervical spine is not necessary in proximal Brachial Monomelic Amyotrophy patients.

Conflict of interest

The author declares no conflict of interest.

ПРОКСИМАЛЬНА ПЛЕЧОВА МОНОМЕЛІЧНА АМІОТРОФІЯ АБО ХВОРОБА ХІРАЯМА: БІЛЬШЕ НЕ ОДНЕ І ТЕ Ж? (КЛІНІЧНИЙ ВИПАДОК)

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Вступ. *Брахіальну мономелічну аміотрофію (БММА) ще називають хворобою Хіраяма, якщо вона характеризується однобічною дистальною слабкістю верхньої кінцівки і атрофією, яка прогресує протягом невеликого періоду часу, а МРТ виявляє типові ознаки у шийному відділі хребта у положенні його згинання.*

Мета роботи *дослідити особливості та відмінності перебігу хвороби при ізольованому ураженні проксимального відділу верхньої кінцівки на прикладі клінічного випадку.*

Методи. *Описано та проаналізовано клінічний випадок брахіальної мономелічної аміоатрофії у дорослого чоловіка, жителя Індії.*

Результати. *30-річний чоловік звернувся зі скаргами на слабкість у проксимальному відділі лівої верхньої кінцівки, яка триває близько чотирьох років. Слабкість прогресувала приблизно півроку після її появи, з того часу – без змін, не спостерігалося ні погіршення ні покращення стану руки. МРТ шийного відділу з контрастом виявила невелике сплощення шийного лордозу, однак не було виявлено типових ознак хвороби Хіраяма: локалізованої вогнищевої атрофії спинного мозку, відшарування задньої стінки дурального мішка від стінок хребцевого каналу в нейтральному положенні на сагітальних T2BI зображеннях, чи наявність додаткових мас-утворів на задній поверхні твердої мозкової оболонки на сагітальних T2BI зображеннях МРТ при згинанні шийного відділу хребта. Пацієнтові було призначено підтримуючу терапію, він знаходиться під регулярним спостереженням лікаря. Клінічний стан стабільний.*

Висновки. *На основі аналізу клінічного випадку, ми підтримуємо пропозицію розглядати брахіальну мономелічну атрофію як окрему нозологічну одиницю, відмінну від хвороби Хіраяма, у пацієнтів з ураженням верхньої кінцівки на підставі результатів обстеження МРТ шийного відділу хребта у положенні згинання.*

КЛЮЧОВІ СЛОВА: **Брахіальна мономелічна аміотрофія; хвороба Хіраяма; МРТ шийного відділу хребта у положенні згинання.**

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EVALUATION OF SERUM γ-GLUTAMYL TRANSFERASE AND ITS ASSOCIATION WITH HIGH SENSITIVITY C-REACTIVE PROTEIN AND INSULIN LEVELS IN THE PATIENTS WITH METABOLIC SYNDROME

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Background. *Metabolic syndrome (MS), a collection of cardiovascular risk factors, is a major worldwide public health problem. The gathered data prove that serum gamma-glutamyl transferase (γGT) activity is a true marker of atherosclerotic cardiovascular disease (CVD) and is of a prognostic importance as well as the high-sensitivity C-reactive protein (hs-CRP).*

Objectives. *In the study, we sought to evaluate serum γGT activity, hs-CRP and insulin resistance in patients with MS.*

Methods*. The study involved 50 persons with metabolic syndrome and 50 healthy age and sex matched controls. Fasting serum samples of all participants were investigated for γGT, hs-CRP, insulin, blood glucose, lipid profile and liver function tests. Anthropometric measurements and BMI were also calculated*

Results. *In that case 50% showed significantly high γGT compared to the controls, 30% proved increased hs-CRP levels above >0.5 mmol/L, whereas 94% of the controls were within the reference range. 74% of cases revealed the presence of insulin resistance while 32% of the controls showed insulin resistance. High γGT levels were also observed in that case with deranged lipids levels and high BMI.*

Conclusions. *The study suggests that the patients with MS have a higher serum γGT activity. This study also proves that hs-CRP and HOMA-IR, which are independent risk factors of CVD, are also associated with MS. The correlation between γGT and the components of MS are also found significant compared to hs-CRP. Thus, γGT can be considered as an inexpensive and authentic predictor of MS, which can be a manifestation of CVD in near future.*

Key words: **metabolic syndrome; gamma-glutamyl transferase; high sensitivity C-reactive protein; HOMA-IR.**

Introduction

Metabolic syndrome (MS) is defined by a constellation of risk factors of cardiovascular disease (CVD), that include abdominal obesity, dyslipidemia, hypertension, and impaired glucose tolerance, which increase the risk of CVD and diabetes mellitus [1]. MS has been considered as one of the threatening non communicable public-health problem globally [2].

Serum gamma-glutamyl transferase (γGT) has long been considered a harbinger of hepatic dysfunction and alcohol intake [3]. Recently, accumulating epidemiology studies have revealed that γGT contributes in several pathophysiological processes, including oxidative stress and lipid peroxidation, which are important for pathogenesis and development of insulin resistance as well as MS [4, 5, 6]. In addition, when compared with other hepatic markers, γGT was the major predictor of type 2 diabetes [7,8,9]. γGT is a possible risk factor and a prognostic indicator of CVD. Further information is needed regarding the magnitude of the risk associated with γGT activity and individual cardiometabolic disorders. Such a relationship could help to decipher a high prevalence of MS.

Perhaps excessive energy consumption, which leads to obesity, is a more serious and frequent nutritional problem, but there can be a gradual and fairly predictable transition from simple obesity with no observable metabolic changes through insulin resistance. Insulin resistance arises from the inability of insulin to act normally in regulating nutrient metabolism in peripheral tissues. Increasing evidences of human population studies and animal research have established correlative as well as causative relations between chronic inflammation and insulin resistance [10]. Chronic, systemic, subclinical inflammation has also been identified as a driving force for insulin resistance. Since

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hs-CRP is a marker of systemic inflammation, it might explain the prevalence of insulin resistance in MS. Nevertheless, the relationship remains uncertain and has not been well researched yet. Therefore, the aim of this study was to examine the associations of serum γGT, hs-CRP and insulin resistance in the individuals with MS as well as its components.

Methods

Source of Data

This study was a hospital based cross sectional study, which comprised metabolic syndrome patients attending the outpatient and inpatient Departments of Medicine. The study was approved by the Local ethical committee of the institute and the informed consents were obtained from all subjects, who took part in the study.

Selection of Subjects

All subjects were diagnosed according to National Cholesterol Education Program, Adult Treatment Panel III criteria and it required the presence of 3 or more of the following [2]: a) fasting blood glucose ≥ 6.105 mmol/L; b) serum triglyceride ≥1.71 mmol/L or being on lipid lowering therapy; c) Serum HDL < 2.220 mmol/L in men and < 2.775 mmol/L in women or being on antilipidemic therapy; d) blood pressure ≥130 mmHg systolic and/or ≥85 mmHg diastolic or being on antihypertensive therapy; and e) waist circumference >102 cm in men and >88 cm in women. The subjects with following history were excluded. Alcohol intake more than 30 g/day (≈38 ml of 100% alcohol) and the patients with smoking history, Hepatitis B or C infection or other known liver diseases, liver enzymes exceeding the upper reference range in three times, use of hepatotoxic drugs, acute infectious/inflammatory conditions, familial hyperlipidemia, New York Heart Association class 3-4 heart failure.

Sample size

After consulting a statistician, sample size was estimated to be 100, with 50 cases and 50 age and sex matched healthy controls.

Type of study: a cross sectional observational study.

Method of sample collection

The informed consents were taken from the patients and control subjects. The selected subject's blood samples were collected with all aseptic precautions. 5 ml of blood was collected from median cubital vein. The collected blood was allowed to clot for 30 minutes in a clean dry test tube and was subjected to centrifugation to separate the serum. The serum samples were stored in a Deep freezer at -80^{0}C till they were studied.

The following parameters were considered appropriate for the study:

1. Serum insulin levels defined by chemiluminescence method and insulin resistance by homeostasis of model assessment of insulin resistance (HOMA-IR).
2. Serum γGT by colorimetric method.
3. hs-CRP by chemiluminescence method.
4. Renal and liver function tests by colorimetric method.
5. Lipid profile by enzymatic, colorimetric method.
6. Fasting blood sugar by hexokinase method.
7. Measurement of body mass index.
8. To measure waist circumference, top of right iliac crest was located. A measuring tape was placed in a horizontal plane around abdomen at level of iliac crest. Before reading the measurements, it was estimated that the tape was snug but did not compress the skin and was parallel to floor. The assessment was performed at the end of normal expiration.

Statistical analysis

Descriptive and inferential statistical analysis has been carried out during the study. The results on continuous measurements are presented on Mean±SD (Min-Max) and the results on categorical measurements are presented in Number (%). Statistical processing of the research results was performed by parametric analysis with the calculation of Student's t-test using the software package Microsoft EXCEL 5.0. Chi-square test was used to find the significance of study parameters on categorical scale between two or more groups. Pearson correlation between γGT and HOMA-IR and hs-CRP were performed to measure the strength between variables and relationships.

Results

The clinical characteristics of the study population are presented in Table 1. The current study is a case control study, in which the serum γGT, hs-CRP and insulin levels were determined in 50 metabolic syndrome subjects and were compared with 50 healthy age and sex matched controls. The results were tabulated and statistically analyzed.

The metabolic syndrome patients were diagnosed according to the National Cholesterol Education Program's Adult Treatment Panel III criteria (NCEP ATP III criteria). The study

Table 1. Clinical characteristics of the study population

Parameters	Controls	**Cases**	**P value**
None of the subjects	50	50	
Sex (male/female)	18/32	20/30	0.68
Age	50.2±9	51.4±9.7	<0.05*
BMI (kg/m^2)	21.5±3.5	29.6±3.9	<0.001**
Waist circumference (cm)	82.5±10.3	104±9.5	<0.001**

Note: the values expressed as mean ± SD. t-test was used for groups' comparison.
The differences in proportions were assessed by means of Chi-square test.
* Suggestive significance (P value P<0.05);
** Strongly significant (P value P≤0.001).

population belonged to age group ranging 40-70 years old, which was similar in the controls as well. The mean±SD of the cases and controls were 51.4±9. 7 years old and 50.2±9 years old respectively, which suggested that metabolic syndrome was prevalent in late middle ages. Waist circumference (WC) and body mass index (BMI) are the two important anthropometric measurements among the various definitions of metabolic syndrome. The study proved the mean±SD for WC in that case as 104±9.5 cm and in the controls as 82.5±10.3 cm. And the mean BMI in that case was 29.58±3.96 kg/m^2 and in the controls – 23.14±2.52 kg/m^2. Both these parameters were significantly higher in the cases with p≤0.001.

The biochemical characteristics of the study population are presented in Table 2. The mean concentration of fasting blood glucose in the controls was 4.1±0.93 mmol/L; in that case it was 6.5±2.1 mmol/L, which was significantly increased in the subjects with MS. Increased triacylglycerols and decreased HDL-cholesterol were potential markers of CVD.

In this study, mean Triglycerides in metabolic syndrome cases was 1.86±0.96 mmol/L and in the controls, it was 1.41±0.8 mmol/L, which was significantly higher. HDL-cholesterol levels in cases were found to be 0.73±0.2mmol/L and 0.96±0.3mmol/L in the controls. The lower HDL-cholesterol levels in that case was found to be significant with p<0.005.

The mean±SD of γGT in that case was 60.96±45.64 U/L and in the controls 29.78±18.01U/L with a P value <0.001**. The mean±SD of serum insulin in that case was 29.34±26.94 μIU/ml and in the controls 11.97±5.98 μIU/ml with P value ≤0.01**. The mean±SD of hs-CRP in that case was 76.2±47.6 mmol/L and in the controls 27.6± 11.4 mmol/L with P value ≤0.001**. The mean±SD of HOMA-IR in that case was 9.44±4.39 and in the controls 2.32±1.48 with P value ≤0.001**.

The comparison of γGT, insulin, hs-CRP, HOMA-IR is presented in Table 3. Pearson correlation was completed to analyse the relationship between γGT, hs-CRP and HOMA-IR in MS cases are as presented in Table 4. γGT

Table 2. Biochemical characteristics of the study population

Variables	**Controls**	**Cases**	**P value**
Glucose (mmol/L)	4.1±0.93	6.5±2.1	<0.01*
Total cholesterol (mmol/L)	3.8±1.16	4.3±1.38	<0.05*
Triglycerides (mmol/L)	1.41±0.8	1.86±0.96	<0.05*
HDL cholesterol (mmol/L)	0.96±0.3	0.73±0.2	<0.05*
Serum albumin (mmol/L)	36.7±8.2	31.8±7.4	<0.01**
Aspartate aminotransferase (U/L)	19.8±7.9	24.26±15	0.06
Alanine aminotransferase (U/L)	17±9.7	22.38±12.1	<0.01**
Alkaline phosphatase (U/L)	71.4±25.6	83.5±33.9	<0.05*
Serum phosphate (mmol/L)	1.1±0.2	0.9±0.2	<0.01**
Serum creatinine (μmol/L)	61.9±26.5	53.5±0.3	<0.01**

Notes: the values expressed as mean SD. T-test was used for groups comparison.
* Suggestive significance (P value <0.05);
* Moderately significant (P value <0.01);
** Strongly significant (P value ≤0.001).

Table 3. Comparison of γ-GT, insulin, hs-CRP, HOMA-IR in both study groups

Parameters	Controls	Cases	P value
γ-GT (U/L)	29.78±18.01	60.96±45.64	<0.001**
Insulin (μIU/ml)	11.97±5.98	29.34±26.94	<0.01*
hs-CRP (mmol/L)	27.6±11.4	76.2±47.6	<0.001**
HOMA-IR	2.32±1.48	9.44±4.39	<0.01*

Notes: values expressed as mean±SD.
Z-test was used for groups comparison *. Suggestive significance (P value <0.05).
* Moderately significant (P value <0.01);
** Strongly significant (P value ≤0.001).

Table 4. Pearson correlation of γ-GT, HOMA-IR, hs-CRP in metabolic syndrome

Parameters	Metabolic syndrome (n=50)	
	r value	P value
γGT vs HOMA-IR	0.26	0.060+
γGT vs hs-CRP	0.252	0.078+
hs-CRP vs HOMA-IR	0.207	0.15

Notes: + suggestive significance.

showed a positive correlation with HOMA IR and hs-CRP which was of suggestive significance.

Discussion

MS comprises a group of atherogenic factors [11]. Besides, the gathered data have reported of many biochemical and anthropometric parameters associated with MS, together with parameters of obesity and products released by adipose tissue, plasma insulin levels, liver enzymes, and CRP [12, 13].

Many epidemiology studies have proved that circulating serum γGT levels may be associated with the evolvement and clinical progression of CVD, even after adjusting for confounding factor like alcohol consumption [14, 15]. Although high levels of γGT have been speculated to be directly atherogenic [16], just like several other biomarkers for MS, a direct causation of atherosclerosis remains to be elucidated. As presented in Table 3, a higher γGT along with insulin resistance levels in MS involves a potentially greater risk for subsequent development of type 2 diabetes.

The increasing evidences have proved that the circulating γGT, which is primarily synthesized from liver, is a key target organ for development of MS. A number of studies have also shown that the serum level of γGT directly correlates with an increased risk of MS [17]. This was evidenced by significant correlations between γGT levels and all MetS components, independently of age and gender, except for blood pressure values [18]. Hardly any studies have proved increased γGT activity in hypertensives, which could be associated with the relation between γGT and MS [19, 20].

The association between the serum γGT and hs-CRP (Table 2), which is, as put forward by Ortega et al. [21], the low-grade inflammation in liver caused by hepatic steatosis in MS, could have caused increase in γGT levels. hs-CRP, an acute-phase reactant of hepatic origin and a sensitive marker for systemic inflammation, predicts the occurrence of diabetes, metabolic syndrome and atherosclerotic diseases in healthy subjects [23]. It has been hypothesized that increased γGT levels might occur before elevation in CRP, and the related oxidative stress would give rise to a subsequent inflam_ matory response [24]. Also, fatty infiltration in liver might have enhanced oxidative stress, leading to glutathione metabolism with com_ pensatory increase in γGT secretion. As γGT activity reflects oxidative stress and inflammation, the increased levels can actively predict the incidence of MS [17].

Many studies have proved the association between the increased γGT and insulin resistance, as well as the subsequent development of type 2 DM [14, 19]. The increase of γGT levels in serum might be as a result of secondary hepatic inflammation [22].

Conclusions

This study suggests that increased gamma-glutamyl transferase activity could be considered as harbinger of low-grade systematic

inflammation and oxidative stress through mediation of glutathione transport. Current study contributes to the increasing number of evidences that gamma-glutamyl transferase estimation in metabolic syndrome, which is simple and inexpensive, could be considered among the strongest serum predictors of insulin resistance, imminent type 2 diabetes and cardiovascular events.

Conflict of interest

The authors declare no conflict of interest.

ВЗАЄМОЗВ'ЯЗОК МІЖ ПОКАЗНИКАМИ ГАММА-ГЛЮТАМІЛТРАСФЕРАЗИ, ВИСОКОЧУТЛИВОГО С-РЕАКТИВНОГО БІЛКА ТА РІВНЯ ІНСУЛІНУ У ПАЦІЄНТІВ З МЕТАБОЛІЧНИМ СИНДРОМОМ

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Вступ. *Метаболічний синдром (МС), як сукупність факторів ризику розвитку серцево-судинних захворювань (ССЗ), є важливою проблемою охорони здоров'я. Отримані дані свідчать про те, що активність сироваткової гамма-глютамілтрансферази (ГГТ), у якості маркера атеросклеротичного процесу при ССЗ, має прогностичне значення, як і показники високочутливого С-реактивного білка (вч-СРБ).*

Мета. *Дослідити активність сироваткової ГГТ, вч-СРБ та інсулінорезистентність у пацієнтів з метаболічним синдромом.*

Методи. *Дослідження включало 50 пацієнтів з МС та 50 здорових осіб. Зразки сироватки були взяті у всіх учасників натщесерце для дослідження активності ГГТ, рівня вч-СРБ, інсуліну, глюкози в крові, оцінки ліпідного профілю та проведення печінкових проб. Також були розраховані антропометричні показники та індекс маси тіла (ІМТ).*

Результати. *У 50% досліджуваних з метаболічним синдромом активність ГГТ була достовірно вищою відносно контрольної групи, у 30% діагностовано підвищений рівень вч-СРБ (вище >0,5 ммоль/л), тоді як показники 94% осіб контрольної групи знаходилися в діапазоні норми. У 74% випадків виявлено наявність інсулінорезистентності у пацієнтів з метаболічним синдромом, тоді як у контрольній групі цей показник склав 32%. Висока активність ГГТ також спостерігалася при порушеннях ліпідного профілю та високому ІМТ.*

Висновок. *Отримані нами дані свідчать, що у пацієнтів з метаболічним синдромом активність сироваткової гамма-глютамілтрансферази достовірно вища, порівняно з контрольною групою. Також нами встановлено, що показники високочутливого С-реактивного білка та рівень інсулінорезистентності, які є незалежними факторами ризику серцево-судинних захворювань, також асоціюються з метаболічним синдромом. Встановлено кореляційні зв'язки між активністю гамма-глютамілтрансферази та високочутливого С-реактивного білка.*

Таким чином, показники сироваткової гамма-глютамілтрансферази можна розглядати як економічно доступний та достовірний предиктор метаболічного синдрому, що може передувати появі серцево-судинних захворювань у найближчому майбутньому.

КЛЮЧОВІ СЛОВА: **метаболічний синдром; гамма-глютамілтрансфераза; високочутливий С-реактивний білок; індекс HOMA-IR (Homeostasis Model Assessment of Insulin Resistance).**

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DEATH DUE TO CARDIAC ANGIOSARCOMA: AUTOPSY CASE REPORT

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Background. *Primary tumors of the heart are rarely detected at autopsy, especially angiosarcomas which are primary malignant one.*
Objective. *We presented autopsy case of cardic angiosarcoma with morphologic findings.*
Methods. *We described adult man died in emergency service of the hospital.*
Results. *Reported case was 33 year-old-man who was died in emergency service of hospital where he was taken when he was ill after leaving home. According the prosecution documents, and the expressions of family, it was reported that he had a heart disease; his symptoms repeated 3 day ago before he died, he thought to attend the Cardiology Clinic due to his symptoms. At autopsy on macroscopic internal examination, mass with rough surface in the right atrium, hematoma at the posterior of the right atrium, blood in the pericardia, nodular lesions in hemorrhagic appearance in the sections of lung, liver and spleen were detected. In histopathologic examination; in the heart angiosarcioma as primary malign heart tumor and metastatic masses in the liver, spleen and lung were detected.*
Conclusions. *We aimed to discuss cardiac angiosarcoma case with autopsy and histopathologic findings in the aspect of medico legal literature.*
KEYWORDS: **Angiosarcomas; heart; metastatic tumors; autopsy.**

Introduction

Primary tumors of heart are rare and they are revealed with prevalence between 0.0017 and 0.19 percent at autopsy [1]. While 75 percent of primary tumors of heart are benign, 25 percent are malign tumors [2]. Angiosarcomas, which are 35-40 percent of primary malign tumors, are the most common [3, 4]. Primary tumors are mostly developing in the right atrium and pericardium [5]. Diagnosis of patients is delayed until tumors become untreatable and many systemic metastases are present [4, 6]. *The research is aimed* to study the autopsy and histopathologic findings of the case in medical and legal aspect.

Case Report

The case involves a 33 year-old-man reported to pass away in the emergency department of hospital, where he was admitted, when he got ill after leaving home. In the analysis of the prosecution documents and according to the words of family members, it was reported that he had a heart disease; chest pain repeated 3 days before he died, he thought to attend the Cardiology Clinic due to the symptoms. In autopsy external examination, there were no pathologic findings, except for ecchymosis due to catheters on the dorsum of the right and left hands and left inguinal line. In internal examination revealed mass on the rough surface of the right atrium (Fig. 1), hematoma at the posterior of the right atrium, 500 cc free blood in pericardia, inhemorrhagic nodular lesions in the sections of lungs, liver and spleen (Fig. 2).

Histopathologically, in sections of heart, tumoral proliferation of atypical cells, which had a high mitotic index and spindle-oval nucleus and infiltrates between myocardial fibers, were observed. There was diffuse hemorrhagic necrosis in the myocardial muscle fibers around tumoral tissue. In lungs, a group of atypical spindle tumoral cells, which had thin-walled vessels and were separated from lung parenchyma with irregular borders, were observed. In liver, many extravasated erythrocytes and tumoral lesions consisting of spindle cells were evidenced. In spleen, many extravasated erythrocytes and tumoral lesions consisting of spindle cells were present. The histopathologic examinationrevealed angiosarcioma in the heart as primary malign heart tumor and metastatic tumors in the liver, spleen and lung. The toxicological investigations were absolutely negative. The cause of death was reported as cardiac angiosarcoma.

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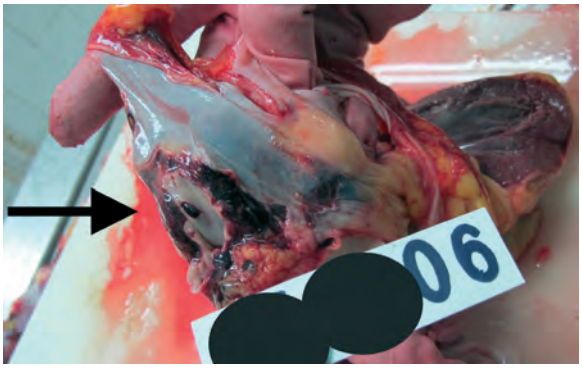

Fig. 1. The arrow shows the mass on the rough surface of the right atrium (arrow).

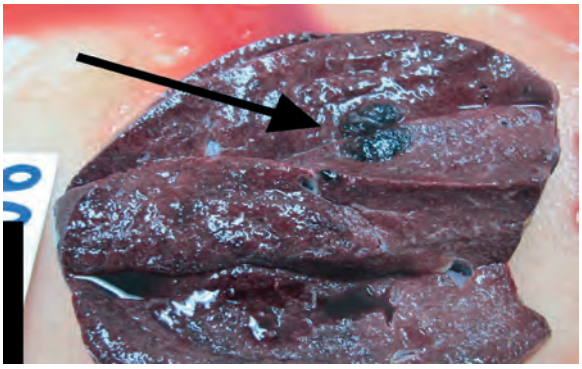

Fig. 2. The arrow shows hemorrhagic nodular metastatic lesions in the sections of spleen (arrow).

Discussion

Primary tumors of the heart are rare and are revealed with prevalence between 0.0017 and 0.19 percent at autopsy [1]. Approximately three-quarters of primary heart tumors are benign with atrial myxomas comprising three-quarters of them [2]. Angiosarcoma is the most common of malignant cardiac tumors [7]. Cardiac angiosarcomas can be found in right atrium, left atrium, pulmonary artery, but most commonly in the right atrium [8], as in our case.

Angiosarcoma is established to be as two-fold in men, typically between the third and fifth decade [4, 10] and our case is consistent with the literature. A study reported that a small series of heart sarcomas were subjected to a specific molecular study that proved the presence of K-*ras* mutations at codon 13 [11]. In human study mutation of the p53 tumor suppressor gene was present in cardiac angiosarcomas [12]. Cardiac sarcomas are rare but lethal disease. It is associated with a poor prognosis and median survival time from 1 to 81 months [6]. Angiosarcoma is associated with a high rate of hematogenous metastases, most of this tumors produce systemic metastases at the time of its detection, most commonly to the lungs, in addition to the liver, brain, adrenal glands, and bone [6, 5, 13]. In our case lung, liver metastases were present compliant with the literature. In addition, in our case there were splenic metastasis different from the literature.

Patients often have non-specific symptoms. So, it is difficult to diagnose cardiac angiosarcoma. Initial findings may include chest pain, dyspnea, fatigue, cough, heart murmur of unclear mechanisms, superior vena cava syndrome, constitutional symptoms, pericardial effusion, pericardial tamponade, arrhythmias, non-specific ST changes on ECG [6, 13, 14] Echocardiography, magnetic resonance image (MRI) and computed tomography (CT) are used for diagnosis and systematic evaluation of cardiac angiosarcoma [15, 16, 17]. Echocardiography, transesophageal or transthoracic, is the main tool for describing the localization and size of a mass [18]. There is no specific tissue density that is why the diagnosis of angiosarcoma is challenging. The histological examination of fluid taken by pericardiosentesis, revealed malignant cells. The definitive diagnosis of angiosarcoma is biopsy [15, 16]. Several studies established that infiltration of the myocardium by spindle cells, hyperchromatic nuclei, mitotic figures in various stages, giant cells, including tumor necrosis and hemorrhage in common areas, negative for cytokeratin, vimentin and FVIII were revealed as positive staining by histopathological examination [6, 15, 16]. Ge et al. reported that the tumor cells in cardiac angiosarcomas were strongly positive for CD31, CD34, FLI-1, and WT-1 but negative for AE1/3, D2-40, human herpesvirus 8, and epidermal growth factor receptor by immunohistochemical method [6].

The rareness of this disease limits gaining experience for adequate treatment. The therapeutic approach for primary cardiac angiosarcoma is surgery, chemotherapy and radiotherapy, alone or in combination. The initial treatment is surgery [6, 19].

Conclusions

Primary tumors of the heart are mostly originating in the right atrium and rarely observed at autopsy. Angiosarcomas are most commonly seen primary malign tumors. We discussed cardiac angiosarcoma case with autopsy and histopathologic findings in the aspect of medico legal literature.

Conflict of interest

The authors declare no conflict of interest.

РАПТОВА СМЕРТЬ ВНАСЛІДОК АНГІОСАРКОМИ СЕРЦЯ: РЕЗУЛЬТАТИ АУТОПСІЇ

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Вступ. *Первинні пухлини серця вкрай рідко зустрічаються при аутопсіях, особливо ангіосаркоми, які належать до злоякісних новоутворів.*

Мета роботи *привернути увагу та ознайомити широке коло читачів з результатами аутопсії та морфологічними даними при ангіосаркомі серця.*

Методи. *Описано та проаналізовано результати аутопсії дорослого чоловіка, який помер у відділенні невідкладної допомоги.*

Клінічний випадок. *33-річний чоловік помер у відділенні невідкладної допомоги, куди був доставлений з дому. Зі слів рідних у нього була хвороба серця, а перед смертю протягом трьох днів турбував біль у серці, з приводу якого він планував звернутися у лікарню. При аутопсії виявлено новоутвір з шорсткою поверхнею у правому передсерді, гематому на задній стінці правого передсердя, 500 мл крові у перикарді, також було виявлено вузлові утвори при розтині легень, печінки та селезінки. За результатами гістологічного дослідження встановлено діагноз ангіосаркоми серця, як первинної пухлини, з метастазами у легені, печінку та селезінку.*

Висновки. *Описано рідкісний клінічний випадок ангіосаркоми серця, виявлений посмертно на аутопсії.*

КЛЮЧОВІ СЛОВА: **ангіосаркома; серце; метастази; ауатопсія.**

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PULMONARY AND INTRACRANIAL RADIOGRAPHIC PRESENTATIONS OF LANGERHANS CELL HISTIOCYTOSIS

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Background. *Langerhans Cell Histiocytosis is a rare disease that affects 1 to 2 adults per million worldwide and often consists of systemic manifestations including pulmonary, intracranial and osteolytic lesions and endocrinologic abnormalities such as Diabetes Insipidus.*

***Objective*.** *The objective of this case report was to expand the medical literature of this rare disease.*

Methods. *A case report of a 51-year-old female patient presenting with systemic symptoms as a result of Langerhans Cell Histiocytosis is presented.*

Results. *A 51-year-old female presented with epistaxis, fatigue, polydipsia, polyuria, headaches and dyspnea. After initial x-rays showed multiple lung and liver nodules and the patient suffered subsequently from a unilateral pneumothorax, an open lung biopsy was recommended. On a pathological basis, the patient was diagnosed with Langerhans Cell Histiocytosis. This report focuses on the radiological presentations of the manifestations of Langerhans Cell Histiocytosis, particularly the presentations in the lung and intracranial regions.*

Conclusions. *Langerhans Cell Histiocytosis is an incredibly rare disease that presents systemically. Recognizing and differentiating radiographic presentation of these patients is important to determine the need for confirmation by biopsy and early chemotherapeutic intervention.*

KEY WORDS: **Langerhans Cell Histiocytosis; FDG-PET (positron emission with ^{18}F-2-deoxy-D-glucose (FDG); MRI (magnetic resonance imaging); X-ray.**

Introduction

Langerhans Cell Histiocytosis is a rare disorder that affects approximately 1 to 2 adult patients per million worldwide [1]. Langerhans Cell Histiocytosis is the accepted terminology as past references of diffuse reticuloendotheliosis, Histiocytosis X, Hand-Schuller-Christian disease and Letterer-Siwe disease are no longer used to refer to this pathology. The Birbeck granule is a characteristic feature of the Langerhans cell, which has several origins including monocytes, langerin positive dendritic cells, monocytes and langerin positive dendritic cells found in the lymphoid tissues [2]. These cells go through an induction process where they resemble the Langerhans cell phenotype [3]. Evidence for the neoplastic argument lays in the MAP2K1 and BRAF mutations in the MAPK pathway [4]. It is a group of disorders of idiopathic pathology although there has been much medical debate as to whether this pathology is neoplastic in nature or inflammatory. The pulmonary form of Langerhans Cell Histiocytosis is prevalent in approximately 10% of cases. In addition, pulmonary Langerhans Cell Histiocytosis expresses greater levels of CD207, S100 and CD1a than other pulmonary diseases [5]. The most common variants of the pulmonary Langerhans Cell Histiocytosis include BRAF V600E and MAPK2K1 [5].

Objective. This case report details clinical evidence and radiographic findings of both the common intracranial and rarer pulmonary form of Langerhans Cell Histiocytosis and is an important work for recognizing presentations of the disease.

Case Report

A 51-year-old Native American female presented to the emergency department with a three-month history of severe headaches, unresolving epistaxis, weakness, mood swings, lower back pain, fatigue, dyspnea, polyuria, polyphagia and polydipsia. The patient's past medical history was positive for depression, nausea, vitiligo and hypothyroidism. She used Synthroid, Folic Acid, Bupropion and Metoclopramide with allergies to Metformin. Family history was positive for hypothyroidism, hypertension, diabetes, congestive heart failure, COPD and coronary artery disease. Surgical history was positive for a hysterectomy, double

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ovarian cystectomy, tonsillectomy and two C-sections. The patient's social history was positive for 25 pack years of tobacco smoking and social alcohol consumption. Physical examination revealed a pulse rate of 88 beats per minute, respiration was 18 breaths per minute, temperature was 37.5 degrees Celsius and blood pressure was 113/75 mmHg.

The decision was made to admit her to the hospital and complete testing secondary to some of the symptoms she was experiencing. X-rays were completed for dyspnea and lower back pain. The chest X-ray visualised multiple liver and lung nodules and MRI of both areas was recommended. The lung nodules were then biopsied endoscopically. Approximately two days after the lung nodule biopsy, the patient suffered a pneumothorax and a needle aspiration was performed. Upon stabilization and subsequent examination by the pulmonologist, an open lung biopsy was recommended to be sent for pathological review to a tertiary care centre. The pathological result showed CD1a, CD207 and BRAF V600E positive tissue. She was diagnosed with Langerhans Cell Histiocytosis and Diabetes Insipidus secondary to the primary diagnosis. An MRI of the brain both with and without contrast was performed where T1 isointense and T2 hyperintense signal areas in the hypothalamus and pituitary stalk were visualized. A 4.5 mm x 4.5 mm x 4.5 mm mass was found in the hypothalamus with an extension into the pituitary stalk. A T2 weighted image showed hyperintensity in the pons area and a thickened and enhanced pituitary stalk. A PET scan was done where diffuse and patchy hypermetabolic activity was found in the liver and spleen consistent with the pathologically confirmed diagnosis of Langerhans Cell Histiocytosis. An MRI of the abdomen showed an intense T2 signal in the portal triads. Centrilobular pulmonary nodules and hypermetabolic activity with lytic lesions in the sacrum and pelvis were found consistent with the diagnosis of Langerhans Cell Histiocytosis confirmed by open lung biopsy. There was the presence of 4 mm stellate nodules, both reticular and nodular opacities and honeycombing at the periphery of the lungs.

Discussion

The hallmarks of Langerhans Cell Histiocytosis are well known. These include lytic bone lesions in particular, which is the most common manifestation [1]. The jaw, vertebra, ribs, digits and skull are commonly involved. There can also be a papular and erythematous rash in the abdominal and groin area that can resemble a candida infection, found particularly in pediatric patients [1]. Oral manifestations of this pathology include ulcers of the soft mucosa, gingivitis and oral cavity masses [6]. Additional symptoms include lymphadenopathy, cystic hepatic lesions, splenomegaly and sclerosing cholangitis [7, 8].

Manifestations of the central nervous system include Diabetes Insipidus secondary to infiltration of the neurohypophysis, paraventricular or supraoptic nuclei as well as neurodegenerative issues affecting cognition and ataxia [9, 10]. Diabetes Insipidus is the most common endocrinological presentation of Langerhans Cell Histiocytosis found in up to half of all cases [11]. This is closely followed by the deficiency of growth hormone [11]. The pituitary seems to be affected in a multitude of ways including vasopressin antibodies or invasion by histiocytes [10]. Diabetes Insipidus can occur months and even years before a formal diagnosis of Langerhans Cell Histiocytosis is given, similar to this patient's case presentation.

The intracranial radiographic changes are important to note in Langerhans Cell Histiocytosis as symptoms such as Diabetes Insipidus can occur for years before diagnosis. Hence, the onus is on the physician to determine the need for further clinical testing based on the radiographic presentation of this disease and implement swift chemotherapeutic interventions. Other cranial radiographic changes to consider include an empty sella, hypertrophied and atrophied, asymmetrical and hyperintense enhancement of the anterior pituitary [12]. The posterior pituitary can show T1W1 hyperintensity while the hypothalamus can show enhancement and lesions [12]. In this particular patient, T2 hyperintense signal areas and T1 isointense areas were seen in the hypothalamus in particular (Fig. 2). These hyperintensities also extended into the pituitary stalk and were consistent with the patient's diagnosis of Langerhans Cell Histiocytosis (Fig. 2). Other radiographic changes in the posterior pituitary in particular, include thickening of the pituitary stalk and also enhancement of that area with an absent T1W1 shortening visible [13]. Studies have shown that manifestations of Langerhans Cell Histiocytosis in the brain tend to occur in those areas without a blood brain barrier such as choroid plexuses, the pineal gland, meninges and circumventricular organs [14].

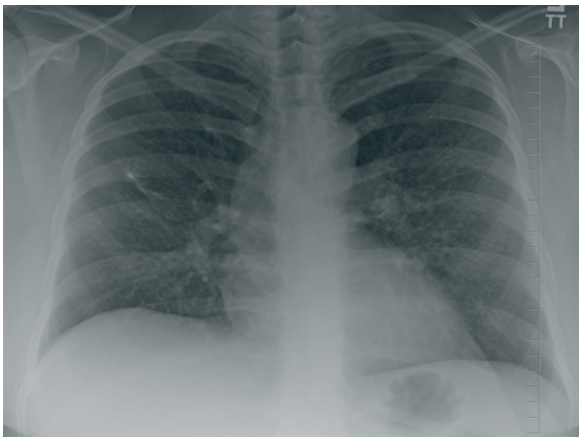

Fig. 1. Chest X-ray exhibiting centrilobular pulmonary nodules and pulmonary cysts with prominence in the bilateral upper lobes and reticular and nodular opacities consistent with Langerhans Cell Histiocytosis.

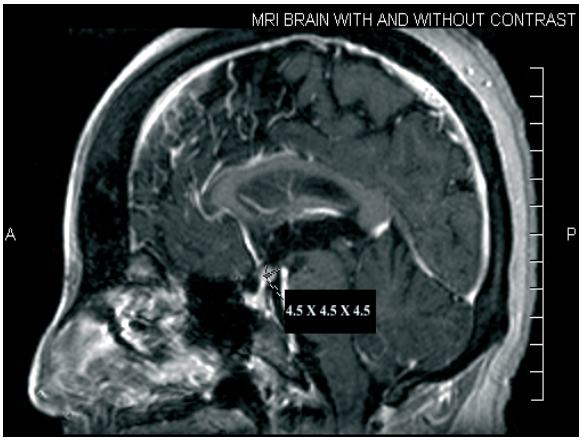

Fig. 2. A 4.5 mm x 4.5 mm x 4.5 mm enhancing hypothalamic mass with extension into the pituitary stalk consistent with Langerhans Cell Histiocytosis.

Neuroendocrine cells of the hypothalamus can project outside of the brain and hence can fall outside the realm of the blood brain barrier even though the actual hypothalamus is protected by one [14].

This case report is important since pulmonary Langerhans Cell Histiocytosis is uncommon and expanding the medical literature on this topic is important for radiologists to determine a possible differential for seemingly common features seen in other diseases such as honeycombing, reticular and nodular opacities. Lung involvement such as this patient's recurrent spontaneous pneumothorax and lung nodules are common in those with Langerhans Cell Histiocytosis [14]. Centrilobular pulmonary nodules with pulmonary cysts as seen in this patient's chest X-rays are radiographic findings that are consistent with an already pathologically diagnosed Langerhans Cell Histiocytosis (Fig. 1). Pulmonary involvement of Langerhans Cell Histiocytosis is a differential when features such as recurrent pneumothorax along with Diabetes Insipidus and bone pain co-occur [15]. Features specific to pulmonary Langerhans Cell Histiocytosis include nodular and reticular opacities, cysts in the upper lung zone along with honeycombing, normal lung volume, no involvement of the costophrenic angle and poorly defined or stellate nodules that may be up to 10 mm in size [16]. Stellate nodules, honeycombing and nodular and reticular opacities were also see in this patient upon histological examination. High resolution CT of the chest can also show interstitial thickening, thin-walled cysts and a general progression from non-cavitating nodules to cavitating nodules and subsequently cystic lesions [17]. Further radiologic presentation of Langerhans Cell Histiocytosis in FDG-PET form is seen by increased uptake in the lungs and nodular radiographic patterns [18].

Conclusions

This case report is important for understanding the simple radiographic presentations of an already rare disease. In conclusion, Langerhans Cell Histiocytosis is an uncommon disease with wide presentations in various organ systems. Radiographic recognition of this pathology can be beneficial to provide further testing and induction of a swift and proper therapeutic regimen.

Conflict of interest

The authors declare no conflict of interest.

ЛЕГЕНЕВІ ТА ІНТРАКРАНІАЛЬНІ РАДІОГРАФІЧНІ ОЗНАКИ ГІСТІОЦИТОЗУ З КЛІТИН ЛАНГЕРГАНСА

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Вступ. *Гістіоцитоз з клітин Лангерганса – рідкісне захворювання, що уражає 1-2 дорослих на мільйон, і часто має системний характер, включаючи остеолітичні прояви та нецукровий діабет серед багатьох інших клінічних симптомів.*

Мета. *Ознайомити читачів та привернути їх увагу до цього рідкісного захворювання*

Методи. *Описано та проаналізовано клінічний випадок гістіоцитозу з клітин Лангерганса з системними проявами.*

Результати. *51-однорічна жінка поступила зі скаргами на носові кровотечі, постійну втому, спрагу, полідипсію та поліурію, головний біль, задишку. При першому рентгенологічному обстеженні виявлено численні вузлики у легенях та печінці, а згодом у пацієнтки розвинувся однобічний пневмоторакс. Після цього було рекомендовано провести біопсію легень. За результатами морфологічного обстеження встановлено діагноз гістіоцитозу з клітин Лангерганса. Описаний нами клінічний випадок фокусує увагу на проявах хвороби, які виявляються при променевих методах обстеження, особливо легень та голови (інтракраніальних ділянок).*

Висновки. *Гістіоцитоз з клітин Лангерганса уражає усі органи та системи організму. Типовими проявами є ураження кісток, розвиток нецукрового діабету внаслідок ураження гіпоталамо-гіпофізарної області, та наявність вузликів у легенях. Зміни, які виявляються променевими методами обстеження, надзвичайно важливі для встановлення потреби у проведенні біопсії та призначення хіміотерапевтичного лікування.*

КЛЮЧОВІ СЛОВА: **Гістіоцитоз з клітин Лангерганса; позитронно-емісійна томографія (ПЕТ) із ^{18}F-ФДГ (18-фтордезоксиглюкоза); МРТ (магнітно-резонансна томографія); рентгенографія.**

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ADIPOSE TISSUE AND ITS ROLE IN MICROENVIRONMENT OF THE COLORECTAL ADENOCARCINOMA CANCER CELL

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Introduction. *The mechanisms of adipose-tissue's influence on tumor progression has been studied a lot, but the way of interaction of adipocytes with tumor cells have not been well defined until now.*

Objective. *The aim of this study was to evaluate the mechanisms of adipocytes and tumor cells interaction under the influence of radiation and chemo-radiation therapy in locally advanced rectal cancer (LARC) patients.*

Material and methods. *A prospective randomized single-center study was conducted. It involved 110 patients with LARC and pre-obesity. The patients were randomized into a main group A (radiation therapy and oxaliplatin-based chemotherapy) and a comparison group B (radiation therapy and fluoropyrimidine-based mono-chemotherapy). Superoxide free radicals and NO levels generated by mitochondria of adipocytes were evaluated In both groups'. Also, there was estimated the indices of MMP-2, MMP-9, 8-oxoG, and free fatty acids (FFA) level.*

Results and discussion. *Level of superoxide radicals in tumor-adjacent adipose tissue was 0.58±0.15 (main group) and 0.70±0.12 nmol/g·min (comparison group) (p<0.001). Blood levels of FFA increased in group A up to 2.05±0.15, and in group B up to 2.48±0.20 mmol/l (while in it was 0.57±0.11 mmol/L). 8-oxoG levels in tumor-adjacent adipose tissue had no statistically significant differences.*

Conclusions. *The tumor-adjacent adipose tissue is an energy depot that can act as a promoter of tumor progression supplying the locally advanced rectal cancer with an energy substrate FFA. It has been established that the level MMP-2 activity significantly reduces the degree of intercellular matrix remodeling by the XELOX chemotherapy.*

KEY WORDS: **locally advanced rectal cancer; adipose tissue; tumor progression; chemoradiotherapy.**

Introduction

A number of clinical studies have proved a direct effect of obesity on the epidemiology of colorectal cancer (CRC), disease progression, and a direct effect of adipose tissue (AT) on malignant neoplasms development, as well as the 'survival' of the latter. Adipocyte is the main structural unit of AT that provides a significant resource of lipids, cytokines and adipokines. It has been established that the lipids, cytokines and adipokines provide regulation of signaling metabolic cascades and spread of malignant cells, including CRC adenocarcinoma cells.

Adipocytes can modify a tumor microenvironment that in its turn stimulates/accelerates the tumor metabolism and leads to formation of an aggressive phenotype of CRC tumor due to paracrine secretion and the presence of significant levels of free fatty acids (FFA). The cells of malignant neoplasms can accelerate the rate of proliferation, which is impossible without the modification of energy metabolism and the initiation of *de novo*-lipogenesis [1]. FFA are used for the accumulation of adipose tissue and the membranes construction, which is the basis for the malignant cells' survival. Most studies have proved that lipids provide survival of adenocarcinoma cells while their own *de novo*-lipogenesis is in a state of inhibition [2]. Thus, it becomes clear that adenocarcinoma cells carry out proliferation without performing the synthesis of their own energy but using energy rich fatty acids of microenvironment [3]. FFA as a powerful source of energy becomes a key element in an aggressive rectal cancer (RC) phenotype formation. It worth remembering that lipids can enhance the Warburg effect in tumor cells [4].

Over the past decade, the awareness of the mechanisms of the AT influence on RC tumor progression has improved considerably. However, the mechanisms of adipocytes and tumor cells interaction under the influence of radiation and chemo-radiation therapy have not been revealed until now.

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Material and methods

A prospective randomized single-center study was conducted. The study involved 110 patients with locally advanced rectal cancer (LARC) (ymrT3-4aN0-2M0-1, CRM-positive) with overweight (pre-obesity), who were treated in National Cancer Institute in January 2016 – December 2018. The patients were randomized into a main group (A) n=57 (radiation therapy with a total focal dose of 50.4 Gy (1.8 Gy×28) and oxaliplatin-based polychemotherapy) and comparison group (B), n=53 (radiation therapy with a total focal dose of 50.4 Gy (1.8 Gy×28) and mono-chemotherapy based on fluoropyrimidine). The patients' division into groups was 1:1. The characteristic features of the groups of patients are provided in Table 1.

In each clinical case, multidisciplinary approach was used, where surgeons, oncologists, chemotherapists and radiologists took part. In all cases, the diagnosis was confirmed cytologically/ histologically after the primary tumor biopsy taken during the colonoscopy in order to assess the resectability of the primary tumor, to detect metastatic lesions in regional lymph nodes and to identify possible distant metastases. A common method of magnetic resonance imaging (MRI) of the small pelvis and abdominal cavity with intravenous contrast and computer tomography (CT) of the chest cavity, abdominal cavity and small pelvis with intravenous contrast, according to international protocols was carried out [5]. Positron-emission tomography was conducted only for planned multivisceral resections and in clinically complicated cases as well as for differential diagnosis for the presence of metastatic lesions of other organs/sites.

Surgical techniques comprised implementation of standardized approaches for RC removing (Total mesorectal excision and D3 lymphatic dissection); most of the interventions were performed laparoscopically.

Sampling of AT was performed for 20 minutes since the removal of the tissue sample at a distance of 1-5 cm from the tumor; a surgeon and a morphologist took part in it. The samples were shaped by a special mold, frozen in liquid nitrogen, with subsequent electron paramagnetic resonance spectra (EPR), they were recorded in a paramagnetic pure quartz Devuar. The levels of superoxide free radicals (SR) generated by mitochondria of adipocytes, as well as the levels of NO content were evaluated by means of EPR method and Spin Traps technology.

The state of intracellular matrix was assessed by the activity of matrix metalloproteinases (MMP-2 and MMP-9) in polyacrylamide gel using zymography technique. After the washing the active forms of MMP-2 and MMP-9 in gel visualized as a discolored stripe on a blue background; their localization was determined according to the molecular weight standards (Sigma) and corresponded to the molecular weight of each of the enzymes (72 and 92 kDa, respectively). The proteolytic activity was evaluated by measuring the area of the lysis zone using a standard set of MMP-2 and MMP-9 (Sigma) for comparison. One a.u. of the enzyme activity assumed as 1 mcg of enzyme in 1 g of control sample. The results were pro-

Table 1. Characteristic features of the groups

Indices	Group A (n=57)	Group B (n=53)	P value
Age	61.4±2.3	64.2±1.9	0.16
Body mass index (BMI)	31.5±2.3	29.2±3.8	0.84
Sex (male/female), (n)	37/21	24/28	0.45
Presence of distant metastases, (n)	14	5	0.19
CRM positive	20	12	0.32
EMVI positive	9	3	0.44
TRG ≥3	10	4	0.25
pN+	28	14	0.21
CEA level	27.4±5.6	12.2±3.1	0.18
Patient's condition (ASA scale):			
I-II	47	39	0.65
III	10	14	0.67

Notes: CRM – 'circumferential resection margin'; EMVI – 'extramural venous invasion'; TRG – 'tumor regression grade'. ASA – American Society of Anesthesiologists scale.

cessed using the standard TotalLab 1.01 program. The levels of DNA guanine oxidation (marker 8-oxoG) in adipose tissue and blood FFA level were determined spectrophotometrically. The content of free (un-esterified) fatty acids (HF) in the blood was determined by spectrophotometric method at λ=546 nm (the intensity of the red dye formed by the interaction of hydrogen peroxide and Tronder compounds is directly proportional to the concentration of non-esterified fatty acids in the knowledge).

The attained results were statistically analyzed by means of SPSS 20.0 statistics (IBM, Armonk, New York, USA). The differences were statistically different at $p<0.05$.

Results

In AT superoxide radicals (SR) and their derivatives are produced by mitochondria and NOX immunocompetent cells. In physiological conditions there is a balance between the SR generation and its elimination by antioxidants. In cases of pathological conditions, in particular, in malignant tumors, there is an increase in SR levels both in tumor and in tissues surrounding it that affects signaling regulatory pathways of tumor cells.

The results of the AT studying are presented in Table. 2. The rate of SR generation in AT stripped at a distance of 1 cm from the macroscopically visible edge of the LARC tumors was determined. In the physiological conditions, the rate of SR generation was 0.18±0.03 nmol/g of tissue·min [6]. The rate of SR generation in tumor-adjacent AT was 0.58±0.15 (group A) and 0.70±0.12 nmol/g tissue·min (group B) ($p<0.001$). The increased blood level of FFA was evidenced in group A: 2.05±0.15 mmol/l, and in group B – 2.48±0.20 mmol/l, the control value was 0.57±0.11 mmol/L. Thus, the damaging effect on the surrounding tissues, in particular in cases of the radiation therapy and administration of platinum, is lower compare to the scheme comprising radiation therapy and fluoropyrimidines. The growth of SR and ROS, respectively, can cause lipids oxidation, 4-hydroxynonenal and malonic dialdehyde formation. The malonic dialdehyde can stimulate the activity of cyclooxygenase-2 (COX-2), which in turn catalyzes synthesis of prostaglandins and induces angiogenesis in RS tumors [7]. Fatty acids, the level of which increases in tumor during their own metabolism, produce electrons; their energy in mitochondrial ETC of tumor cells transform into ATP and SR due to fact that adipocytes mitochondria are dysfunctional (Table 2).

Oxidative DNA damage is also a result of unregulated increase of SR and ROS levels and their effects on human cells. The most common product of the pathological effect of SR on a DNA molecule is 8-oxoguanine (8-oxoG) formation; the level of it starts to exceed the normal values already in the early stages of the disease [8]. 8-oxoG can be accumulated both in nuclear and mitochondrial DNA. That is why they are considered to be a highly informative marker for the development of malignant neoplasms and their progression.

According to our previous studies, 8-oxoG levels in tumor-adjacent AT proved to have no statistically significant differences: 0.80±0.08 and 0.84±0.13, respectively for groups A and B, $p=0.052$ (Table 1 that may be explained by the fact that medicines used for hemotherapy lead to genotoxic effects). Platinum containing medicines can in addition initiate SR generation.

Nitric oxide (NO) is a pleiotropic, regulatory signaling molecule that is crucial for a variety of biological processes, including vasodilation, neurotransmission, and immune response [9]. NO is synthesized out of L-arginine by NO synthase (NOS) [10]. The increased expression of inducible NOS (iNOS) in tumor cells proves direct proportional correlation with survival of patients with RC and other malignant neoplasms of epithelial origin [11]. The activity of iNOS in tumor-adjacent adipose tissue is determined in accordance with the procedure described above. Normal value of the NO content is 1.16±0.20 nmol/g of tissue. Direct evaluation of NO level is 0.51±0.11 and 0.38±0.08 nmol/g of

Table 2. Comparison of the studied molecular markers between groups of patients.

Values	Norm values (Mean±SE)	Group A (Mean±SE)	Group B (Mean±SE)	t-value	P-value
Speed of SR generation, nmol/g tissue•min	0.18±0.03	0.58±0.15	0.70±0.12	4.43	<0.001
NO content level, nmol/g tissue	1.16±0.20	0.51±0.11	0.38±0.08	-7.24	<0.001
8-oxo-G level, nmol/g tissue	0.21±0.03	0.8±0.08	0.84±0.13	1.96	0.052
FFA levels, mmol/L	0.57±0.11	2.05±0.15	2.48±0.20	2.36	0.045

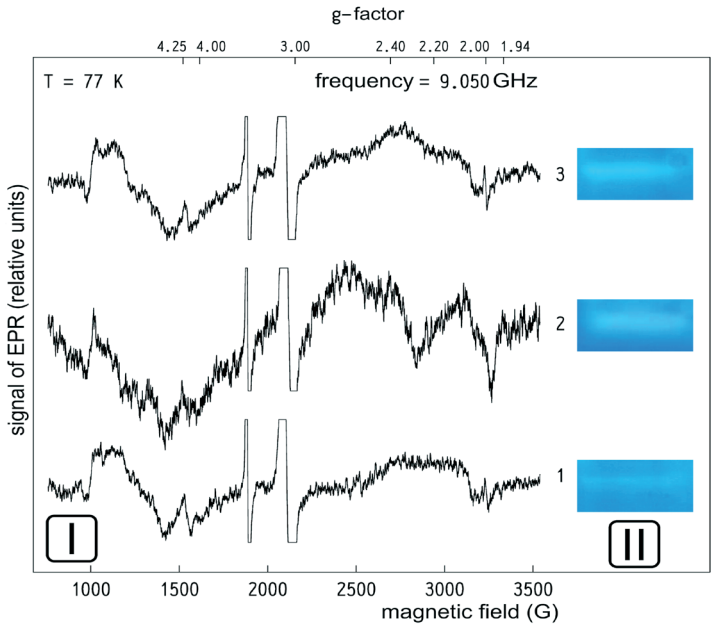

Fig.1. Graphic representation of EPR spectra in studied groups of patients.
I – EPR spectra of AT in patients with RC:
1 – AT of the patients with BMI 25.0–30.0.
2 – group B patients with BMI ≥25.
3 – group A patients with BMI ≥25.
II – zymograms showing MMP-2 activity in AT of the corresponding groups.

tissue in group A and B, respectively ($p<0.001$) (Table 2) that indicates a disturbance of NO bioavailability in tumor-adjacent AT.

The changes of AT cells mitochondrial ETC in the patients with RC in cases of different treatment regimens were revealed. In EPR spectra of AT intensive EPR signals with g=2.03 were recorded that proved formation and increase of NO-FeS proteins complexes in Complex I (ETC) of adipocytes mitochondria in the group A (Fig. 1, EPR 3 spectrum, g=2.03). The level of NO-FeS proteins complexes was 0.27±0.14 a.u. This value in norm was 0.08±0.05 a.u. In the group B of patients, who received fluoropyrimidines instead of oxaliplatin, the level of NO-FeS proteins complexes in ETC were significantly higher (0.36±0.09 a.u.) that indicated that fluoropyrimidines-based AT formed hypoxic microenvironment, which was more favorable for the tumor. The level of NO-FeS proteins complexes in ETC of adipocytes mitochondria correlates with the degree of RC differentiation ($r=0.79$, $p<0.05$). It should also be noted that the level of MMP-2 activity in AT was significantly lower in the group A, indicating that oxaliplatin had less significant effect on redox state of AT and subsequently the intracellular matrix remodeling.

Tissue intracellular matrix remodeling, including degradation and reorganization of its structures developed with the participation of specific enzymes: matrix metalloproteinases (MMP). MMPs are a family of Zn^{2+} and Cu^{2+}-dependent endopeptidases with a common functional domain and activation mechanism.

At present, more than 20 types of MMPs different by their cellular localization, substrate specificity, functional activity, have been established: gelatinase, collagenase, stromelysines, membrane bound MMP as well as a group of insufficiently studied MMP [12].

The increased level of gelatinase subgroup members (MMP-2 and MMP-9) proves the presence of tissue matrix damage. The factors activating the inactive forms of MMP (pro-MMP) are intensification of SR generation in endothelial cells mitochondria and NOX immunocompetent cells of AT; SR reacts with NO forming $ONOO^-$ as a result that leads to disorder of NO bioavailability in AT [11, 13]. It was already noted that in the patients, involved into the research, independently from the treatment method a decrease in the NO content in AT adjusted to tumor was evidenced (Table 2).

In Fig.2 the results of the gelatinases activity (MMP-2 and MMP-9) in the patients with RC is presented.

It was proved that MMP-2 activity rates were (3.51±1.06) and (5.72±1.13) at $p<0.001$, respectively in groups A and B. MMP-9 activity

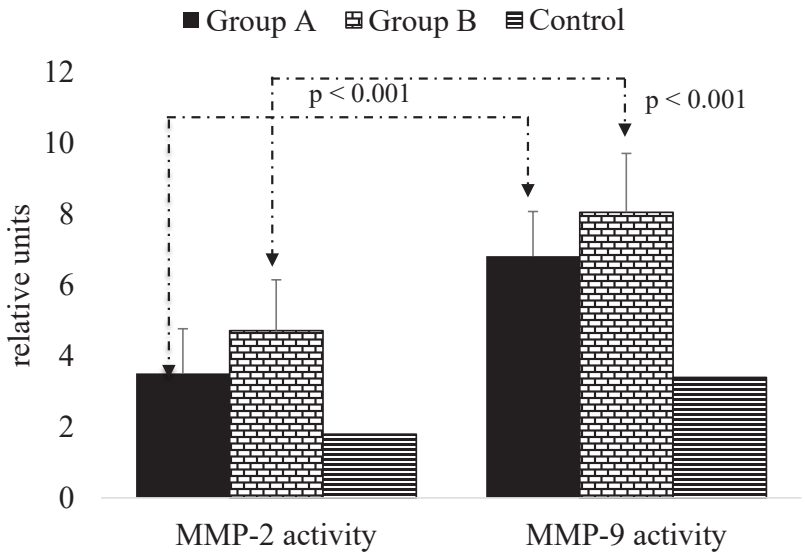

Fig. 2. MMP-2 AND MMP-9 activity in AT of the patients.

rate was (6.82±1.03) and (8.06±1.36) in the main and control groups, respectively (p<0.001). These results indicate that in the group A patients, who received oxaliplatin or capecitabine, the level of intercellular matrix degradation was lower compare to the group B patients administered with fluoropyrimidines.

Discussion

Redox molecules are capable of inducing lipolysis in adenocarcinoma cells that results in glycerol production, which can be incorporated into the glycolytic metabolic pathway [14]. Such pathological changes in normal metabolism can activate more rapid growth and subsequently much significant malignant metastatic tumor phenotype, including RC [15]. Such changes are accompanied by changes in the microenvironment of healthy cells, infiltration of tumor-associated immune cells that trigger the process of chronic inflammation and enhance oxidative stress. The analysis proves that activation of *de novo*-lipogenesis in most of the patients with CRC, is caused by high levels of CR and, consequently leads to MMP activation. The MMP activation, in its turn, proves that the adipocytes of tumor-adjacent AT in the patients with RC are an 'additional' source of energy involved in metabolic processes of the RC adenocarcinoma that contributes to their faster growth and formation of metastases.

In addition, a significant difference in the studied molecular markers proved a promising use of a modified scheme of preoperative course of chemotherapy-based oxaliplatin and capecitabine (XELOX). We believe that such approach can help reduce the percentage of distant metastasis and local relapses.

Conclusions

Tumor-adjacent AT is an energy depot that can act as a promoter of tumor progression, supplying the RC tumor with the energy substrate – FFA. In addition, the oxaliplatin based chemotherapy reduces the level of DNA oxidative-induced point mutations in adipocytes mitochondria. It has been established that the level MMP-2 activity significantly reduces the degree of intercellular matrix remodeling by the XELOX chemotherapy.

Conflict of interest

The author declares no conflict of interest.

ЖИРОВА ТКАНИНА ТА ЇЇ РОЛЬ В МІКРООТОЧЕННІ КЛІТИН АДЕНОКАРЦИНОМИ КОЛОРЕКТАЛЬНОГО РАКУ

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Вступ. *За останні десять років наше розуміння механізмів впливу жирової тканини (ЖТ) на пухлинне прогресування значно покращилось, однак до цього часу не розкрито механізми взаємодії адипоцитів з клітинами пухлин раку прямої кишки (РПК) за умов проведення променевої та хіміотерапії*

Мета. *Дослідити механізми взаємодії адипоцитів та клітин аденокарциноми за умов променевого та хіміопроменевого лікування у хворих на місцево поширені форми раку прямої кишки.*

Методи. *Було проведено проспективне рандомізоване одноцентрове дослідження. У дослідженні брали участь 110 хворих на місцево-поширений рак прямої кишки (мпРПК), (ymrT3-4aN0-2M0-1, CRM – positive) з надлишковою вагою в період з січня 2016 р. до грудня 2018 р. Пацієнтів рандомізували у співвідношенні 1:1 на основну групу (А) n=57 (променева терапія сумарною осередковою дозою 50,4 Гр (1,8 Гр х 28) та поліхімотерапія на основі оксаліплатини), та на групу порівняння (Б), n=53 (променева терапія сумарною осередковою дозою 50,4 Гр (1,8 Гр х 28) та монохіміотерапія на основі фторпіримідинів). У обох групах вивчали рівні мітохондріальних супероксидиних радикалів та NO в адипоцитах прилеглої до пухлини жирової тканини. Також досліджували рівень матриксних металопротеїназ (ММП-2 та ММП-9), маркери окисного пошкодження ДНК (8-oxoG) та рівень жирних кислот.*

Результати. *Зареєстровані рівні швидкості генерування супероксидних радикалів (СР) в ЖТ, яка контактувала з пухлиною складали 0,58±0,15 (група А) та 0,70±0,12 нмоль/г тканини•хв (група Б) (p<0,001). В крові цих хворих виявлено зростання рівня вільних жирних кислот (ВЖК): у групі А до значень 2,05±0,15 ммоль/л, а в групі Б цей показник склав 2,48±0,20 ммоль/л при значеннях норми 0,57±0,11 ммоль/л. Рівні 8-oxoG в прилеглій до пухлини ЖТ хворих досліджуваних груп не мали статистично значущої відмінності – 0,80±0,08 та 0,84±0,13 відповідно для груп А та Б, при p = 0,052.*

Висновки. *Прилегла до пухлини ЖТ представляє собою енергетичне депо, здатне виступати в ролі промотора пухлинного прогресування, забезпечуючи пухлину мпРПК енергетичним субстратом – ВЖК. Було показано, що хіміпроменева терапія (XELOX) знижує рівень ремоделювання міжклітинного матриксу прилеглої жирової тканини.*

КЛЮЧОВІ СЛОВА: **місцево-поширений рак прямої кишки; жирова тканина; пухлинне прогресування; хіміопроменева терапія.**

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SIGNIFICANCE OF ADDITIONAL CHROMOSOMAL ABNORMALITIES FOR THE OUTCOMES AFTER THE SECOND LINE NILOTINIB THERAPY IN THE CHRONIC MYELOID LEUKEMIA PATIENTS

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Background. *There is limited information about impact of additional chromosome aberrations (ACA) on the efficacy of the 2nd line nilotinib therapy.*

Objective. *The aim of the study was to analyze significance of ACAs for the outcome after second line tyrosine kinase inhibitors (TKI) therapy with nilotinib in the chronic myeloid leukemia (CML) patients, who experienced previous imatinib therapy failure.*

Methods. *The CML patients in chronic phase treated with nilotinib after imatinib failure were analyzed for outcomes.*

Results. *Among a total of 114 patients, 18 patients (15.8%) had ACAs at the beginning of the 2nd line therapy with nilotinib. Seven patients (38.9%) of 18 had variant translocations and 11 patients (61.1%) had other chromosomal abnormalities in addition to t(9;22), known as clonal evolution. Complete cytogenetic response (CCR) at 12 months was achieved in 37.5%, 42.8% and 45.5% (p=0.842) of patients with classic t(9;22) translocation, variant translocations and ACAs respectively. In the patients with variant translocations t(9;V;22) or clonal evolution treated with nilotinib after the imatinib failure, the CCR and major molecular response (MMR), event free survival (EFS), progression free survival (PFS) and overall survival (OS) rates did not differ from those in the CML patients with t(9;22) only. At the same time quantitative characteristics of leukemic and ACA clones had prognostic value for CCR. The increased number of Ph-positive cells and the number of cells with the ACA at the start of nilotinib therapy reduced the probability of CCR.*

Conclusions. *Higher nilotinib inhibitory activity compare with imatinib allows us to overcome imatinib resistance in the CML patients regardless of the ACA presence at the beginning of nilotinib therapy.*

KEY WORDS: **additional chromosomal aberrations; chronic myeloid leukemia; nilotinib; second line of TKI therapy; prognosis; resistance.**

Introduction

Reciprocal translocation t(9;22)(q34;q11.2), also known as Ph-chromosome, leads to the formation of the *BCR/ABL1* fusion gene, which is considered to be the main pathogenetic event of chronic myeloid leukemia (CML) [1].

However, additional chromosomal abnormalities along with the classical translocation t(9;22) have been described in some patients with CML. These are usually either variant translocations or additional chromosome aberrations (ACAs). In case of variant translocation (t(9;V;22)), more than two chromosomes are involved in the formation of t(9;22). ACAs include any numerical or structural chromosome aberrations in addition to t(9;22) and are called clonal evolution. At the time of diagnosis, the frequency of ACAs is 5-10% in the patients with chronic phase CML and increases up to 30% and 80% in the patients with acceleration phase or blast crisis [2, 3]. A *de-novo* gain of ACA during the therapy is also possible. It is established that ACAs confer a BCR/ABL-independent mechanism of resistance to the therapy with tyrosine kinase inhibitors (TKI) [4]. Although, currently, there is no final consensus about the predictive value of ACAs, most authors point out that the outcome of imatinib-treated patients with ACAs is significantly lower than of those without ACAs [5, 6].

It has been proved that nilotinib (the second generation TKI) is effective in patients with CML after imatinib failure [7, 8]. But the influence of nilotinib on leukemic clones with additional chromosomal abnormalities has not been determined.

The objective of this study was to analyze the significance of ACAs for the outcome after second line TKI therapy with nilotinib in the CML

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patients, who experienced previous imatinib therapy failure.

Methods

A retrospective study of 114 CML patients in chronic phase treated with nilotinib after imatinib failure was conducted. All patients were monitored at the Department of Radiation Onco-Hematology and Stem Cell Transplantation of the Institute of Clinical Radiology at the National Research Center for Radiation Medicine from 2008 to 2018. Written informed consents to use biomaterial for the research purposes were obtained from all patients. Among a total of 114 patients, 49 (43%) were males and 51 (57%) – females. Imatinib for the first line TKI therapy was administrated at a dose of 400 mg/d with later escalation to 800 mg/d. The second line TKI therapy was implemented with nilotinib dose 400 mg twice a day.

Cytogenetic analysis was conducted at the time of imatinib resistance conformation. Karyotyping with standard GTG-banding was performed after a 24-hour bone marrow cells culture with no stimulation. Chromosomes were classified according to the International System for Human Cytogenetic Nomenclature [9].

Molecular response was assessed in 3, 6 12 months and then every 6 months during nilotinib therapy by real-time polymerase chain reaction as previously reported [10]. A complete cytogenetic response (CCR), major molecular response (MMR) and deep molecular response (MR4) were defined as *BCRABL1/ABL* ratios 1%, 0.1%, and 0.01% respectively, on the international scale. The first and second line TKI therapy responses and resistance were defined according to the ELNet 2013 recommendations [6].

The χ^2 test and Fisher's exact test were used to compare categorical variables and the Mann–Whitney U-test – to compare continuous variables. The probability of overall survival (OS), progression free survival (PFS), and event free survival (EFS) were estimated by the Kaplan-Meier method and compared by the log-rank test. PFS was defined as survival without signs of progression (transformation into acceleration phase or blast crisis). EFS was evaluated from the beginning of the 2nd line therapy with nilotinib to the loss of achieved CCR or MMR, progression or death for any cause. OS was assessed from the beginning of the 2nd line therapy till the death of any cause during the study. The cumulative probability of CCR and MMR was evaluated by the Kaplan–Meier method from the beginning of the 2nd line therapy to the time of response. In this case, the data about the patients, who had not received any adequate response, with progressed disease or died, were censored at the latest observation date. Univariate analyses (Cox regression) were performed to evaluate the prognostic significance of additional chromosomal abnormalities. Statistical analysis was performed using the SPSS for Windows statistical software package (version 20.0). The results were considered significant at $p<0.05$.

Results

The baseline characteristics of the patients are summarized in Table 1.

Among 114 patients, who started the second line therapy with nilotinib, 101 (88.9%) were resistant to previous imatinib therapy. Resistance was defined as failure to achieve CCR on the first line of therapy. Twelve patients (10.5%) achieved only CCR, while 1 (0.9%) patient achieved MMR with previous imatinib therapy. Any achieved therapy response was completely lost in all patients with CCR and MMR by the start of nilotinib. Pathologic clone with classical t(9:22)(q34;q11) was detected in 5-100% of cells of all patients at the time of the

Table 1. CML patients' characteristics at the start of the 2nd line therapy with nilotinib

Patient characteristics	Patients with only t(9;22) (n=96)	Patients with t(9;V;22) (n=7)	Patients with ACA (n=11)	p
Age, median (range), years	38 (18–66)	38 (34–50)	35 (20 – 52)	0.512
Sex, men, n (%)	40 (41.7)	3 (42.9)	6 (54.5%)	0.950
Median of prior imatinib treatment, (range), months	44.0 (4.0–106.0)	39,0 (13.0–48.0)	45.0 (5.0–106.0)	0.578
Median of follow-up on the 2nd line therapy with nilotinib, (range), months	52.0 (4.0–106.0)	42.0 (5.0–97.0)	32.0 (1.0–79.0)	0.194

Notes: CML – chronic myeloid leukemia; ACA – additional chromosomal aberration

second-line therapy initiation. The median number of Ph-positive cells with variant translocation or with an ACA was 15% (range of 5-100%). The median duration of prior imatinib treatment was 43 months (range of 4 to 106 months), while the median duration of the second-line therapy monitoring was 47 months (range of 1 to 106 months).

Among a total of 114 examined patients, 18 patients (15.8%) had ACAs at the beginning of the 2nd line therapy with nilotinib. Seven patients (38.9%) of 18 had variant translocations and 11 patients (61.1%) had other chromosomal abnormalities in addition to t(9;22). Among the other 11, 8 patients had a single chromosomal abnormality in addition to t(9;22), while 3 patients had multiple chromosomal abnormalities. In the majority of patients with ACAs (7 out of 11, 63.6%), an additional translocation was a second Ph-chromosome (Table 2). The patients with an isolated t(9;22) translocation, variant translocations or ACA were not significantly different by age, sex, and length of treatment before the beginning of the second-line therapy.

In order to evaluate the effect of ACAs on response to the second-line TKI therapy, the extent of tumor clone reduction in 12 months of therapy, the probability of CCR and MMR, as well as long-term outcomes (EFS, PFS and OS) were evaluated in all 114 patients with CML, treated with nilotinib after imatinib failure.

The presence of ACAs and variant translocations at the beginning of the 2nd line therapy did not affect the rate of the tumor clone reduction. The CCR in 12 months (optimal response according to ELNet2013) was achieved in 37.5%, 42.8% and 45.5% (p=0.842) of patients with classic t(9;22) translocation, variant translocations and ACAs respectively. In 12 months, about 50% of patients were resistant to the second line TKI therapy (BCR/ABL>10% in 12 months of the therapy according to ELNet2013). The rate of resistance did not differ between the groups: 51.0%, 57.1% and 54.5% in the patients with classic t(9;22) translocation, variant translocations and ACAs, respectively (Table 3).

No differences in the rates of disease progression and lethal outcomes were found

Table 2. Karyotypes of bone marrow cells with variant translocations and additional chromosomal aberrations in the CML patients at the beginning of nilotinib therapy (n=18)

№	Sex	Number of Ph+ metaphases, %	Number of metaphase with ACA, %	Karyotype
1	F	95	100	46,XX,**t(9;22;?)(q34;q11;?)**[19] 46,XX[1]
2	F	100	100	46,XX,**t(5;9;22)(q31;q34;q11)**[20]
3	F	100	5	46,XX,t(9;22)(q34;q11)[19]/idem,**+der(22)t(9;22)** [1]
4	M	73	53,3	46,XY,t(9;22)(q34;q11)[3]/idem,+**der(22)t(9;22)**[8]/46,XY[4]
5	M	100	100	46,XY,**t(6;9;22)(p22;q34;q11)**[20]
6	F	100	100	46,XX,der(9)**t(9;?;22)**(q34;?;q11)[20]
7	M	85	5	46,XY,t(9;22)(q34;q11)[5]/ idem,+**der(22)t(9;22)** [1]/46,XY[14]
8	M	100	70	46,XY,t(9;22)(q34;q11)[6]/idem,+**der(22)t(9;22)**[11]/ idem,+**der(22)t(9;22)**,+**7**[3]
9	M	100	100	47,XY,t(9;22)(q34;q11),+**der(22)t(9;22)**[14]/ idem,+**12**[2]
10	F	50	42,5	47,XX,t(9;22)(q34;q11),+**der(22)t(9;22)**[17]/ 46,XX,t(9;22)(q34;q11)[13]/46,XX[20]
11	M	30	30	46, XY,**t(3;9;22)(p25;q34;q11)**[6] /46,XY[14]
12	F	100	30	47,XX,t(9;22)(q34;q11),+**3**[6]/ 46,XX,t(9;22)(q34;q11)[14]
13	M	20	100	46~48,XY,t(9;22)(q34;q11)[4],**del(10)(p11.2)**[5], +**12**[2],+**13**[12],+**14**[2]+**15**[4] [cp20]
14	F	100	100	46,XX,**t(3;21)(q26.2;q22)**,t(9;22)(q34;q11)[20]
15	M	100	100	46,XY,**t(9;21;22;)(q34; q22.2;q11)**[20]
16	F	100	5	46,XX,t(9;22)(q34;q11)[19]/idem,+**der(22)t(9;22)**[1]
17	M	90	5	46,XY,t(9;22)(q34;q11)[17]/ idem,+**der(22)t(9;22)**[1]/46,XY[2]
18	F	100	100	46,XX,**t(1;9;22)(q21.2;q34;q11)**[20]

Notes: ACA – additional chromosomal aberration.

Table 3. Response to the 2nd line nilotinib therapy in the CML patients depending on the presence of additional chromosomal aberrations

Characteristics	Patients with only t(9;22) (n=96)	Patients with t(9;V;22) (n=7)	Patients with ACA (n=11)	p
Response in 12 months of the 2nd line nilotinib therapy:				
CCR (BCR/ABL1≤1%), n (%)	36 (37.5)	3 (42.8)	5 (45.5)	0.842
MMR (BCR/ABL1≤0.1%), n (%)	13 (13.5)	1 (14.3)	3 (27.3)	0.516
BCR/ABL1>10%, n (%)	49 (51.0)	4 (57.1)	6 (54.5)	0.938
Secondary resistance:				
MMR loss, n (%)	4/30 (13,3)	0/2 (0)	1/5 (20)	0.829
CCR loss, n (%)	4/46 (8.7)	0/4 (0)	0/5 (0)	0.656
Progression to AP or BC, n (%)	19 (19.8)	1 (14.3)	2 (18.2)	0.934
Number of lethal outcomes, n (%)	17 (17.7)	1 (14.3)	1 (9.1)	0.756

Notes: CML – chronic myeloid leukemia; ACA – additional chromosomal aberration; CCR – complete cytogenetic response; MMR – major cytogenetic response, AP – acceleration phase; BC – blast crisis.

between the patients with classic t(9;22) translocation, variant translocations and ACAs (Table 3). For the whole follow-up period, the only case of progression to accelerated phase with a lethal outcome took place in one patient with variant translocation of unknown origin t(9;?;22)(q34;?;q11) and in one patient with three related clones: 30% metaphases with isolated Ph-chromosome, 55% metaphases with an additional Ph-chromosome, and 15% of metaphases with two Ph-chromosomes and an extra chromosome 7. Karyotype 46,XY,t(9;22)(q34;q11)[6]/idem,+der(22)t(9;22)[11]/idem,+der(22)t(9;22),+7[3].

Kaplan-Meier analysis of the long-term outcomes of the 2nd line nilotinib therapy proved an absence of significant influence of the ACA and variant translocation status at the beginning of the 2nd line nilotinib therapy on the three-year EFP, PFS and OS (Table 4).

Despite the ACAs at the beginning of the 2nd line therapy did not affect the nilotinib efficacy, quantitative characteristics of the leukemic clone did have prognostic value. A positive correlation between the amount of Ph-positive clone before the beginning of the 2nd line nilotinib therapy and development of resistance (BCR/ABL1>10%) in 12 months of the subsequent therapy (Spearman correlation coefficient r=0.423, p<0.001) was found. The increase in the number of Ph-positive cells at the beginning of the nilotinib therapy and increase in size of the additional clone (percent metaphases with ACA) was associated with the decreased efficiency of tumor clone reduction during the therapy, or more specifically, statistically significant decrease in the rate of CCR (p<0.001, p=0.049). In other words, the efficacy of the second line TKI therapy in the imatinib-resistant CML patients was associated with the level of reduction of the tumor clone and the size of the clone with additional aberrations before the beginning of the second-line therapy (Table 5).

Discussion

Studies of the ACA prognostic value both in the era before the TKI and after approving of the first TKI imatinib indicate that the ACA

Table 4. Long-term outcomes of the 2nd line nilotinib therapy in the CML patients depending on the presence of additional chromosomal aberrations

Outcomes	Patients with only t(9;22) (n=96)	Patients with t(9;V;22) (n=7)	Patients with ACA (n=11)	p
3-year EFS, % (95% CI)	80.1 (71.5–88.7)	80.0 (45.5–100.0)	74.1 (42.5–100.0)	0.468
3-year PFS, % (95% CI)	86.4 (79.1–93.7)	80.0 (45.5–100.0)	76.2 (47.2–100.0)	0.936
3-year OS, % (95% CI)	90.7 (84.6–96.8)	80.0 (45.5–100.0)	85.7 (59.8–100.0)	0.975

Notes: CML – chronic myeloid leukemia, ACA – additional chromosomal aberration, CI – confidence interval, EFS – event free survival, PFS – progression free survival, OS – overall survival.

Table 5. Estimation of the significance of ACA clones quantitative characteristics in the CML patients (n=18) at the beginning of the 2nd line nilotinib therapy for the probability of CCR, EFS, PFS and OS by the univariate Cox regression analyses

Characte-ristics	CCR		EFS		PFS		OS	
	Exp(B) (95% CI)	p	Exp(B) (95% CI)	p	Exp(B) (95% CI)	p	Exp(B) (95% CI)	p
% Ph+ metaphases	0.981 (0.971–0.990)	<0.001	1.027 (1.00–1.054)	0.039	1.037 (1.000–1.076)	0.052	1.062 (0.997–1.132)	0.062
% meta-phases with ACA	1.041 (1.000–.083)	0.049	1.028 (0.979–1.080)	0.268	1.031 (0.979–1.085)	0.248	1.025 (0.973–1.080)	0.354

Notes: CML – chronic myeloid leukemia; ACA – additional chromosomal aberration; CI – confidence interval; EFS – event free survival; PFS – progression free survival; OS – overall survival.

presence in the therapy worsens the treatment results and decreases patient survival [11–13]. Some disagreements are observed in assessment of the ACA significance at the time of diagnosis. Alhuraiji A., et al. in the prospective clinical study on 603 CP CML patients pointed out that the presence of ACA at the time of diagnosis was not associated with worse prognosis [14]. Fabarius, et al. consider that only major route, but not balanced or unbalanced minor route ACA has a negative impact on prognosis of CML [13]. Wang et al. also underline that variability in outcomes is related to the type of ACA, but they suggest another ACA stratification system. 'Poor prognosis' for ACA including i(17)(q10), -7/del7q, and 3q26.2 rearrangements as well as presence of 2 or more ACAs conferred a worse survival irrelevant to the emergence phase and time. In contrast, ACAs in group of 'good prognosis' including trisomy 8, -Y, and an extra copy of Ph-chromosome had no adverse impact on survival, when they emerged at the chronic phase or at the time of CML diagnosis [15].

Nilotinib is more potent and selective ATP-competitive inhibitor of BCR/ABL1 tyrosine kinase than imatinib and it is more active against imatinib-resistant mutant forms of enzyme [16, 17]. Multicenter trials and a real-life longitudinal proved nilotinib efficacy in the CML patients with imatinib failure [8, 18].

Our report presents outcomes after the second line therapy with nilotinib in the CML patients with imatinib failure depending on the presence of variant translocations or clonal evolution at the beginning of the 2nd line therapy with nilotinib. Among a total of 114 examined patients, 18 patients (15.8%) had clonal evolution at the beginning of nilotinib. Seven patients (38.9%) of 18 had variant translocations and 11 patients (61.1%) had clonal evolution – additional to t(9;22) chromosomal abnormalities. Our results suggest that for the patients with variant translocations t(9;V;22) or clonal evolution treated with nilotinib after imatinib failure, the CCR and MMR response, EFS, PFS and OS rates do not differ from those of the CML patients with t(9;22) only. At the same time quantitative characteristics of leukemic and ACA clones have a significant impact on CCR. The greater number of Ph-positive cells and the number of cells with the ACA are revealed at the beginning of nilotinib, the less – for CCR probability. EFS is also worse with increasing number of Ph-positive cells at the beginning of nilotinib. The significance of certain types of ACA for long-term outcomes has not been investigated because of small groups of patients with different ACAs.

Conclusions

The presence of ACA provides the tumor with heterogeneous properties that determine its sensitivity to therapy. But higher inhibitory activity of nilotinib compare to the imatinib allows overcoming imatinib resistance in the CML patients regardless of the ACA presence and achieves CCR in about 50 % of patients in 12 months of nilotinib therapy. Investigation of prognostic value of the combination ACA with other tumor clone features advantages development of criteria for the individual selection of TKI in the resistant patients.

Conflicts of Interest

Authors declare no conflict of interest.

ЗНАЧЕННЯ ДОДАТКОВИХ ХРОМОСОМНИХ АБЕРАЦІЙ У ФОРМУВАННІ ВІДПОВІДІ НА ДРУГУ ЛІНІЮ ТЕРАПІЇ НІЛОТИНІБОМ У ХВОРИХ НА ХРОНІЧНУ МІЄЛОЇДНУ ЛЕЙКЕМІЮ

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ДУ "НАЦІОНАЛЬНИЙ НАУКОВИЙ ЦЕНТР РАДІАЦІЙНОЇ МЕДИЦИНИ НАЦІОНАЛЬНОЇ АКАДЕМІЇ МЕДИЧНИХ НАУК УКРАЇНИ", КИЇВ, УКРАЇНА

Вступ. *Існує обмежена інформація про вплив додаткових хромосомних аберацій (ДХА) на ефективність терапії нілотінібом другої лінії у хворих з хронічною мієлодною лейкемією (ХМЛ).*

Мета: *проаналізувати значущість ДХА щодо відповіді на терапію інгібіторами тирозинкіназ (ІТК) другої лінії (нілотинібом) у хворих на ХМЛ, у яких терапія іматинібом була неефективною.*

Методи. *Аналізували ефективність терапії нілотинібом у хворих на ХМЛ в хронічній фазі, у яких терапія іматинібом була неефективною.*

Результати. *Серед 114 пацієнтів 18 пацієнтів (15,8%) мали ДХА на початку 2-ї лінії терапії нілотінібом. Сім пацієнтів (38,9%) з 18 мали варіантні транслокації, а 11 пацієнтів (61,1%) мали інші хромосомні аномалії, крім t(9;22), тобто ознаки клональної еволюції. Повна цитогенетична відповідь (ПЦВ) через 12 місяців терапії нілотинібом була досягнута у 37,5%, 42,8% і 45,5% (p=0,842) пацієнтів з класичною транслокацією t(9;22), варіантними транслокаціями та клональною еволюцією відповідно. У пацієнтів з варіантнимии транслокаціями t(9;V;22) та клональною еволюцією швидкість ПЦВ та великої молекулярної відповіді (ВМВ), безподійна виживаність (EFS), виживаність без прогресії (PFS) та загальна виживаність (OS) не відрізнялися від таких у хворих на ХМЛ, у яких виявлялась тільки транслокація t(9;22). В той же час, кількісні характеристики лейкемічного клону та клону з ДХА мали прогностичне значення щодо досягнення ПЦВ. Збільшення кількості Ph-позитивних клітин та клітин з ДХА на початку терапії нілотинібом зменшувало вірогідність досягнення ПЦВ.*

Висновки. *Більш висока інгібуюча активність нілотинібу в порівнянні з іматинібом дозволяє подолати резистентність до іматинібу у пацієнтів з ХМЛ незалежно від наявності ДХА на початку терапії нілотинібом.*

КЛЮЧОВІ СЛОВА: **додаткові хромосомні аберації; хронічна мієлоїдна лейкемія; нілотиніб; друга лінія терапії ІТК (інгібітори тирозинкіназ); прогнозування; резистентність.**

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MORTALITY ANALYSIS OF THE PATIENTS WITH ALCOHOLIC LIVER CIRRHOSIS

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Background. *Alcohol is considered to be the main risk factor for adverse event deaths around the world. In Ukraine, mortality due to alcoholic liver disease (ALD) has taken the second place in the structure of death causes from diseases of the digestive system.*

Objective. *The aim of the research was to study the peculiarities of the causes of death in the patients with alcoholic liver disease at the stage of liver cirrhosis (LC) based on the analysis of protocols of pathoanatomical research.*

Methods. *The analysis of 149 protocols of the pathoanatomical study of the patients, who died from alcoholic LC, has been carried out at the premises of the Pathoanatomical Department of the Ivano-Frankivsk Regional Clinical Hospital in 2006-2018.*

Results. *Most people were young and middle aged. Fatal cases were caused by decompensation of the LC with the development of hepatic, hepatic-renal, cardio-pulmonary insufficiency, pancreatic necrosis, gastrointestinal bleeding (GIB), sepsis, hepatocellular carcinoma (HCC). In 37.6 % of the patients the concomitant illness was coronary heart disease (CHD), 10.7 % of the people had hypertension. In 6 % of the patients, ischemic stroke of the brain was diagnosed. In most people atherosclerotic vascular changes were revealed.*

Conclusions. *Excessive consumption of alcohol and, consequently, the development of LC, can be considered as an adverse factor in the reducing social standard of living. In the majority of people, who died from the decompensation of alcoholic LC, atherosclerotic vascular lesions have been detected. This indicates a significant prevalence of lipid metabolism disturbance in the people with alcoholic LC.*

KEY WORDS: **liver cirrhosis, alcoholic; cause of death; coronary disease; atherosclerosis.**

Introduction

Demographic processes in Ukraine have signs of a protracted demographic crisis, which is closely related to the historical and socio-economic peculiarities of the country's development. It is well-known that Ukraine belongs to countries with a progressive decline in demographic and reproductive potential, which leads to depopulation and population decline. At the beginning of 2016, the number of children in the population of Ukraine was 16.1 %, those of working age – 61.8 %, older than able-bodied – 22.1 %. The prevalence of women in the elderly as a result of higher mortality among males is the peculiarity of gender imbalance. Demographic aging, that is, the steady increase in the proportion of economically and socially inactive elderly people, combined with a decrease in the proportion of able-bodied people, has a direct impact on various spheres of life in the Ukrainian society [1, 2].

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Along with the aging of the population, the increase in the mortality rate, which is associated with endogenous diseases (circulatory and neoplastic diseases) and exogenous (respiratory diseases, digestive disorders, infectious and parasitic diseases, external) causes, is a topical issue. In recent years, much attention has been paid to the increase in the incidence of so-called non-infectious diseases, which is becoming a sign of not only an epidemic, but also a possible future pandemic [3, 4, 5]. According to the WHO, 41 million people die from these diseases every year, accounting for 71% of all deaths in the world. Annually 15 million people between 30 and 69 years of age die from them. Cardiovascular diseases (stroke, heart attack) and malignant neoplasms are leading in prevalence and mortality among non-infectious diseases [6, 7].

Non-communicable diseases are the result of the combination of genetic, physiological, environmental and behavioural factors. Behavioural factors belong to the modified factors, and they can be changed. These include the use of tobacco, lack of physical activity, inappropriate nutrition, and alcohol abuse. The other not less

important factors include metabolic factors: increased blood pressure, overweight/obesity, hyperglycemia, hyperlipidemia [8, 9, 10].

Among non-infectious diseases caused by these risk factors, the digestive diseases together with the increase of the proportion of deaths are significant. Fibrosis and LC, alcohol and non-alcoholic liver diseases, which accounts for more than 50 % of those who died of digestive diseases, contributed much to mortality from this class of deaths causes. The reasons for this are the continuous increase of quantitative and qualitative types of such patients, frequent chronic diseases, prolonged and severe courses, unfavourable consequences of a disease, prevailing affection of people of working age that is associated with medical and socio-economic factors [11, 12, 13].

The main causes of liver damage are alcohol, viruses, non-alcoholic fatty liver disease. Alcohol is considered to be the main risk factor for adverse lethal outcomes around the world. Alcohol abuse is third among the causes of mortality among young people after tobacco and arterial hypertension and secondarily among the causes of liver transplantation in Europe. In Ukraine, mortality due to ALD takes the second place in the structure of causes of death from digestive diseases [14, 15, 16].

The aim of the research was to study the peculiar features of the death causes in the patients with ALD at the stage of LC based on the analysis of protocols of pathoanatomical investigation.

Methods

The analysis of 149 protocols of pathoanatomical research of the patients, who died from alcoholic LC at the premicies of the Pathoanatomical Department of the Ivano-Frankivsk Regional Clinical Hospital in 2006-2018, was conducted. The average age of patients was (56±12.1) years old (y. o.): women – (44.6±9.2) y. o., men – (57.6±11.8) y. o., the average duration of the disease was (6.6±1.8) years. The patients were divided into groups by age as follows: 44 young people (31 males and 13 females), 76 middle-aged persons (64 males, 12 females), 29 elderly patients (19 males, 10 females).

Results

According to the Child-Pugh criteria, among the patients with the LC, who died, there are 9 (6%) persons with stage A of the disease, 26 (17.5%) – with stage B, 114 (76.5%) persons – with stage C. The causes of death in the patients with the LC of alcoholic etiology were pancreatic necrosis (4.7 % of patients with stage A, 8.8 % of patients with stage B and 1.3 % of patients with stage C), gastrointestinal bleeding (GIB) – in 4 % of patients with stage B and in 36.3 % of patients with stage C, liver failure – in 17.4 % of patients with stage C, liver and kidney failure – in 1.3 % of people with stage B and in 10.7 % of persons with stage C, sepsis – in 1.3 % of patients with stage B and in 6.7 % of patients with stage C, cardio-pulmonary insufficiency – in 1.3 % of patients with stage A and 2 % of patients with stage B, HCC – in 4 % of people with stage C (Table 1).

According to the medical records, in all patients who died, the signs of portal hypertension, hepatosplenomegaly, cytolytic, hepatodepressive, mesenchymal-inflammatory syndrome were revealed. Among the manifestations of hepatic hyperazotemia there were hepatic encephalopathy and hepatopulmonary syndrome in all patients and hepatorenal syndrome – in 87.9 % (3.4 %, 10.7 %, 4.7 % of cases in young, middle aged and elderly patients), respectively. The signs of cholestasis syndrome were detected in 88.6 % of patients.

Excessive subcutaneous fat was in 34.9 % of cases, satisfactory – in 42.3 %, insufficient – in 22.8 %. 134 (89.9 %) patients had ascites; 18.1

Table 1. Death causes in the patients with liver cirrhosis

Death causes	Stage of Child-Pugh criteria					
	A (n=9)		B (n=26)		C (n=114)	
	n	%	n	%	n	%
Pancreatic necrosis	7	4.7	13	8.8	2	1.3
Gastrointestinal bleeding	-	-	6	4	54	36.3
Liver failure	-	-	-	-	26	17.4
Liver and kidney failure	-	-	2	1.3	16	10.7
Sepsis	-	-	2	1.3	10	6.7
Cardio-pulmonary insufficiency	2	1.3	3	2	-	-
Hepatocellular carcinoma	-	-	-	-	6	4

% of them suffered from ascites-peritonitis. Septicemic condition was revealed in 8.7 % of cases and steatonecrosis of the omentum – in 16.1 % of people. Hydrotorax was diagnosed in 83.2 % of cases, hydropericardium – in all cases. Varicose of the oesophagus veins was diagnosed in all the lethal outcomes: of the 1st stage – in 9.4 % (14 persons), 2nd stage – in 20.8 % (31 persons), 3rd stage – in 69.8 % (104 persons) of cases, varicose veins of the stomach were present in 40.9 % of cases. The bleeding from varicose veins of the oesophagus was in 57.7 %, a combination of bleeding from the veins of the oesophagus and the stomach – in 30.2 % of cases. Depending on the age, the GIB was in 10.7 %, 18.8 %, 6.7 % of the patients of young, middle and old age, respectively. Among men, GIB was revealed in 35.5 % of young people, in 35.9 % of middle-aged patients, in 15.4 % of the elderly. Among women, GIB was revealed in 38.5 % of young people, in 41.7 % of middle-aged patients and 30 % of the elderly (Table 2).

Erosive gastroduodenitis was revealed in 66.4 % (99 persons) of cases, acute ulcers of the duodenal bulb (DB) – in 10.7 % (16 persons), acute ulcers of the stomach – in 2.7 % (4 persons). The umbilical hernia was present in 12.7 % of cases. Chronic hemorrhoids were diagnosed in 10.7 % of people (with bleeding in 4.7 % of cases).

Mild anemia was evidenced in 14.1 % of patients, moderate – in 39.6 %, severe – in 49.7 %. In 86.6 % of cases, the signs of chronic pancreatitis were revealed, 55 % of patients had pancreatic lipodystrophy, 14.8 % suffered from pancreatic necrosis (young and middle-aged persons), 7.4 % had cholelithiasis, 61.7 % – the signs of chronic cholecystitis, in 2 % of people the signs of cholangitis were present. Hypotonia of the gall bladder was revealed in 12.1 % of all cases.

Type 1 diabetes mellitus was diagnosed in 2 % of young people, type 2 – in 4.7 % of middle-aged and elderly people. Chronic pyelonephritis

Table 2. The revealed pathoanatomical changes of internal organs depending on the liver cirrhosis decompensation stage

Pathological changes of internal organs	Stage by Child-Pugh criteria					
	A		B		C	
	n	%	n	%	n	%
	9	6	26	17.5	114	76.5
Cardiovascular system:						
- hydropericardium	9	6	26	17.5	114	76.5
- hypertension	6	4	8	5.4	2	1.3
- IHD	3	2	22	14.8	31	20.8
- atrial fibrillation	-	-	3	2	2	1.3
- ischemic stroke of the brain in anamnesis	3	2	6	4	-	-
- chronic venous insufficiency of the vessels of the lower extremities	-	-	-	-	7	4.7
- atherosclerosis of the coronary arteries	9	6	24	16.1	22	14.8
-- stage of the lipid stain	2	1.3	10	6.7	5	3.4
-- stage of atherosclerotic plaque	5	3.4	10	6.7	17	11.5
-- complicated plaque with ulceration	-	-	4	2.7	-	-
-- narrowing of LCA by 10%	3	2	3	2	2	1.3
-- narrowing of LCA by 30%	3	2	2	1.3	2	1.3
-- narrowing of LCA by 50%	-	-	3	2	-	-
- atherosclerosis of the iliac arteries	4	2.7	8	5.3	5	3.4
-- stage of the lipid stain	-	-	6	4	5	3.4
-- stage of atherosclerotic plaque	4	2.7	2	1.3	-	-
- atherosclerotic changes in the aorta	9	6	26	17.5	84	56.4
-- stage of the lipid stain	2	1.3	9	6	36	24.2
-- stage of atherosclerotic plaque	4	2.7	12	8	42	28.2
-- complicated plaque with ulceration	3	2	5	3.4	6	4
Respiratory system:						
- hydrothorax	-	-	10	6.7	114	76.5
- pulmonary edema	9	6	26	17.5	114	76.5
- pulmonary emphysema	-	-	2	1.3	4	2.7
- pneumonia	-	-	7	4.7	98	65.8
- pleurisy	-	-	6	4	35	23,5
- CORP	-	-	3	2	8	5.4

Pathological changes of internal organs	Stage by Child-Pugh criteria					
	A		B		C	
	n	%	n	%	n	%
	9	**6**	**26**	**17.5**	**114**	**76.5**
Digestive system:						
- portal hypertension	9	6	26	17.5	114	76.5
- cytolytic syndrome	9	6	26	17.5	114	76.5
- hepatodepressive syndrome	9	6	26	17.5	114	76.5
- mesenchymal-inflammatory syndrome	9	6	26	17.5	114	76.5
- cholestasis syndrome	-	-	18	12.1	114	87.9
- ascites	-	-	20	13.4	114	86.6
- ascites-peritonitis	-	-	-	-	27	18.1
- steatonecrosis of the omentum	-	-	-	-	24	16.1
- splenomegaly	9	6	26	17.5	114	76.5
- umbilical hernia	-	-	-	-	19	12.7
- varicose of the esophagus veins	9	6	26	17.5	114	76.5
1-st degree	9	6	5	3,4	-	-
2-nd degree	-	-	21	14.1	10	6.7
3-d degree	-	-	-	-	104	69.8
-- with bleeding	-	-	-	-	86	57.7
- varicose veins of the stomach	-	-	-	-	61	40.9
-- with bleeding	-	-	-	-	45	30.2
- erosive gastroduodenitis	-	-	8	5.3	91	61.1
- acute ulcers of the duodenal bulb	-	-	-	-	16	10.7
- acute ulcers of the stomach	-	-	-	-	4	2.7
- chronic hemorrhoids	-	-	3	2	13	8.7
- hemorrhoidal bleeding	-	-	-	-	7	4.7
- chronic pancreatitis	-	-	15	10.1	114	76.5
- pancreatic lipodystrophy	-	-	6	4	76	51
- pancreatic necrosis	10	6.7	8	5.4	4	2.7
- cholelithiasis	2	1.3	5	3.4	4	2.7
- chronic cholecystitis	-	-	16	10.7	76	51
- cholangitis	-	-	-	-	3	2
- hypotonia of the gall bladder	-	-	-	-	18	12.1
- type 1 diabetes mellitus	-	-	-	-	3	2
- type 2 diabetes mellitus	-	-	2	1.3	5	3.4
Urinary system:						
- chronic pyelonephritis	-	-	2	1.3	12	8.1
- kidney cyst	-	-	-	-	3	2
Nervous system:						
- encephalopathy	9	6	26	17.5	114	76.5
- alcoholic delirium in anamnesis	7	4.7	4	2.7	-	-

was present in 9.4 % of cases, kidney cyst – in 2 %.

Pneumonia was diagnosed in 70.5 %, pleurisy – in 27.5 %, chronic obstructive pulmonary disease (COPD) – in 7.4 %, pulmonary emphysema – in 4 %. In 4.7 %, chronic venous insufficiency of the vessels of the lower extremities was revealed.

Among the concomitant diseases, hypertension was present in 10.7 % of middle aged and elderly patients, ischemic heart disease (IHD) – in 37.6 % of cases, 3.4 % of these patients suffered from atrial fibrillation. 6 % of patients had ischemic stroke of the brain in anamnesis, and 7.4 % – alcoholic delirium. The signs of atherosclerosis were also evidenced. Particularly, the biochemical parameters of the lipid spectrum were characterized by an increase in the content of total cholesterol in the blood, low and very low-density lipoprotein cholesterol. Moreover, the degree of increase of these indicators was directly proportional to the degree of decompensation of LC.

Atherosclerotic changes in the aorta at the stage of lipid stain were present in 31.5 % of cases, at the stage of atherosclerotic plaque – in 38.9 %, at the stage of complicated plaque with ulceration – in 9.4 % of people. Atherosclerosis of the left coronary artery (LCA) was revealed in 11.4 % at the lipid staining stage, at the stage of atherosclerotic plaque – in 21.5 %, ulcerated atherosclerotic plaque was present in 2.7 %.

Narrowing of LCA by 10% was revealed in 5.3 % of cases, by 30 % – in 4.7 %, by 50 % – in 2 %. Atherosclerosis of iliac arteries was present in 11.4 % of patients.

Discussion

Consequently, analyzing clinical records of the pathoanatomical study of the patients with alcoholic LC, it was established that most patients were young and middle aged and died from decompensated LC with the development of multiple organ failure. In all cases, there was a lesion of the pancreas, in half of them lipodystrophy was revealed. Bleeding from varicose veins of the esophagus and stomach was the cause of death in 57.5 % of people, 14.8 % of patients died from pancreatic necrosis, 4 % – from HCC. IHD was the concomitant illness in 37.6 % of the patients. More than 70 % of the dead had atherosclerotic lesions of aorta, and one third of the persons had injured coronary vessels.

Excessive consumption of alcohol and, consequently, the development of LC, can be considered as an adverse factor in the reducing social standard of living. Fatal cases were caused by decompensation of LC, development of multiple organ failure, pancreatic necrosis, bleeding from the esophagus and stomach veins, HCC. Special attention should be paid to the combination of ALD with atherosclerotic vascular changes revealed in 79.8 % of people, indicating a significant prevalence of lipid metabolism disorders among people with decompensated alcoholic LC.

Consequently, analyzing clinical records of the pathoanatomical study of the patients with alcoholic LC, it was established that most patients were young and middle aged and died from decompensated LC with the development of multiple organ failure. In all cases, a lesion of the pancreas was present, in half of them pancreatic lipodystrophy was revealed. The cause of death in 40.3 % of people was bleeding from varicose veins of the esophagus and stomach with subcompensation and decompensation of LC, 17,4 % of patients died from hepatic failure with decompensation of LC, 13.5 % of patients died from pancreatic necrosis with compensation and subcompensation of LC. Hepatic-renal failure was cause of death of the patients with subcompensation and decompensation of LC. Sepsis caused death of 8 % of people with subcompensation and decompensation of LC, 4 % of patients died from HCC. Cardio-pulmonary failure caused death in the patients with compensation and subcompensation of LC.

The cytolytic, hepato-depressive, mesenchymal-inflammatory, hepatic encephalopathy, cholestatic, hepatorenal, anemic, hepatopulmonary syndromes and portal hypertension were especially significant in the patient with subcompensation and decompensation of LC.

More than a third of people had overdeveloped subcutaneous fat. IHD was the concomitant illness in 37.6 % of the patients. More than 70 % of the dead had atherosclerotic lesions of aorta, and one third of the persons had injured coronary vessels.

Excessive consumption of alcohol and, consequently, the development of LC, can be considered as an adverse factor in the reducing social standard of living. Fatal cases were caused by decompensation of LC, development of multiple organ failure, pancreatic necrosis, bleeding from the esophagus and stomach veins, HCC. Special attention should be paid to the combination of ALD with atherosclerotic vascular changes revealed in 79.8 % of people, indicating a significant prevalence of lipid metabolism disorders among people with decompensated alcoholic LC.

Conclusions

Analyzing clinical records of the pathoanatomical study of the patients with alcoholic liver cirrhosis, it was established that most patients were young and middle aged and died from decompensated LC with the development of multiple organ failure. Bleeding from varicose veins of the esophagus and stomach were the causes of death in 57.5 % of people, 14.8 % of patients died from pancreatic necrosis, 4 %– from HCC. IHD was the concomitant illness in 37.6 % of the patients. More than 70% of the dead had atherosclerotic lesions of aorta, and one third of the persons had injured coronary vessels. Pancreatic necrosis and cardio-pulmonary failure were the causes of death in most people with stages A and B. GIB, hepatic and hepatic-renal failure, sepsis, and development of HCC were the causes of death in most people with stage C. IHD was the concomitant illness in 37.6 % of the patients.

Conflict of interest

The author declares no conflict of interest.

АНАЛІЗ СМЕРТНОСТІ ХВОРИХ НА АЛКОГОЛЬНИЙ ЦИРОЗ ПЕЧІНКИ

Н.Р. Матковська
ДВНЗ "ІВАНО-ФРАНКІВСЬКИЙ НАЦІОНАЛЬНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ", ІВАНО-ФРАНКІВСЬК, УКРАЇНА

Вступ. *Алкоголь визнають основним фактором ризику несприятливих летальних завершень у всьому світі. В Україні смертність внаслідок алкогольної хвороби печінки (АХП) посіла друге місце в структурі причин смерті від хвороб органів травлення.*

Мета. *Метою було вивчити особливості причин смерті у осіб з АХП на стадії цирозу печінки (ЦП) на основі аналізу протоколів патологоанатомічного дослідження.*

Методи. *Проведено аналіз 149 протоколів патологоанатомічного дослідження померлих на алкогольний ЦП на базі патологоанатомічного відділення Івано-Франківської обласної клінічної лікарні за період 2006-2018 рр.*

Результати. *Більшість осіб були молодого та середнього віку. Летальні випадки були зумовлені декомпенсацією ЦП з розвитком печінкової, печінково-ниркової, серцево-легеневої недостатності, панкреонекрозу, шлунково-кишкових кровотеч, сепсису, гепатоцелюлярної карциноми. У 37,6% померлих супутньою хворобою була ішемічна хвороба серця, у 10,7% осіб – гіпертонічна хвороба. У 6% осіб в анамнезі був перенесений ішемічний інсульт головного мозку. У більшості осіб виявлено атеросклеротичні зміни судин.*

Висновки. *Надмірне вживання алкоголю і, як наслідок, розвиток цирозу печінки можна вважати несприятливим чинником зниження рівня здоров'я населення. У більшості осіб, що померли внаслідок декомпенсації алкогольного виявлено атеросклеротичні ураження судин. Це вказує на значну поширеність порушення ліпідного обміну у осіб з алкогольним цирозом печінки.*

КЛЮЧОВІ СЛОВА: **алкогольний цироз печінки; причини смерті; ішемічна хвороба серця; атеросклероз.**

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PROBING BREAST CANCER THERAPEUTIC RESPONSES BY DNA CONTENT PROFILING

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Background. *Discrepancies in the interpretation of breast cancer therapeutic responses still exist mainly because of lack of standardized assessment criteria and methods.*

Objective. *DNA content profiling of cells in the affected (cancerous) tissue before and after neoadjuvant chemotherapy (NAC) was applied to facilitate interpretation of therapeutic responses.*

Methods. *Both diagnostic biopsy and operation materials representing the tissue of primary tumors surgically removed after NAC were subjected to DNA image cytometry. Polyploidy and aneuploidy in DNA histograms were evaluated with a prognostic Auer typing. Stemline DNA index (DI) values and percentages of cells that polyploidize (>4.5C) were also determined. Immunofluorescence staining was applied to evaluate proliferation (Ki-67), invasiveness (CD44), and self-renewal factors characteristic for stem cells (SOX2 and NANOG).*

Results. *DNA content profiles of 12 breast cancer cases, of which 7 were triple-negative, revealed the features of tumor non-responsiveness to NAC in 7 cases, of which 5 were triple-negative. Among non-responsive cases there were 3 cases that showed enhanced polyploidization, suggesting the negative NAC effect. Near-triploid (DI=1.26-1.74) triple-negative cases were determined as most resistant to NAC. Cycling near-triploid cells may contribute to the excessive numbers of >4.5C cells. Polyploid cells were positive for Ki-67, CD44, SOX2, and NANOG.*

Conclusions. *DNA content profiling data provide additional helpful information for interpreting therapeutic responses in NAC-treated breast cancers. Polyploid tumor cells possessing stem cell features can be induced by NAC. Because NAC effects in some cases may be unfavorable, the use of the further treatment strategy should be carefully considered.*

KEY WORDS: **breast cancer; polyploid cells; near-triploidy; DNA content profiling; therapeutic response; cancer stem cells.**

Introduction

Neoadjuvant therapy that was initially designed for the preoperative treatment of patients with locally advanced breast cancer [1] presently provides a good opportunity to evaluate therapeutic response of primary tumors and customize subsequent conventional non-surgical treatment [2–5]. Therapeutic response is usually assessed by means of histological examination of the affected breast tissue stained with hematoxylin and eosin. To date, most histological criteria consider the absence of invasive cancer cells in the breast and regional lymph nodes as a pathologic complete response (pCR) [3, 4]. Contrary to the patients, who have partial or no response, those patients, who achieve pCR, largely have favorable prognosis (improved long-term, disease-free, and overall survival) [5]. Although the histological assessment of therapeutic responses significantly helps to determine prognosis in breast cancer, the problem caused by discrepancies in the interpretation of results obtained from different pathologists remains unresolved mainly because of lack of standardized assessment criteria and methods [4, 5]. Moreover, the term pCR still needs to be applied in a consistent, standardized manner [6].

Breast cancer as many other solid tumors is predisposed to high degrees of aneuploidy (abnormal chromosome number) [7, 8] that correlates with the resistance to anti-cancer treatment and poor prognosis [9]. To minimize misinterpretations in histopathologic scoring of therapeutic responses, DNA content profiling of cells in the affected breast tissue before and after neoadjuvant therapy seems to be a good option since this technique is capable of detecting tumor cell DNA aneuploidy that may

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often be unseen by histopathologists. In other words, the advantage of the technique is in detection of very small numbers of aneuploid tumor cells in a large cohort of analyzed cells. In this regard, the intra-tumor genetic diversity that can potentially be manifested in clonal heterogeneity is worth mentioning [10, 11]. There are situations when in a tumor the drug-sensitive dominant clones can be eliminated, while the minor resistant clones or sub-clones can be hiding in a dormant state followed by accelerated cell proliferation. Also, to date, the issue of detecting and quantifying the resistant tumor cells that polyploidize in response to genotoxic therapies is of pivotal importance [12, 13]. Cytometric determination of DNA content in tissue specimens for detecting malignancies, monitoring responses to therapy, and prognosing disease outcome is very important and demanding [12, 14]. Notably, cell nuclei isolated from aneuploid-gated distributions by means of fluorescence activated cell sorting can be subjected to single-cell DNA and RNA sequencing to study breast cancer chemoresistance evolution [15].

In this study, the image cytometry-based DNA content profiling of cells of the affected breast tissues of women predominantly with locally advanced Stage III disease before and after neoadjuvant chemotherapy (NAC) with paclitaxel and doxorubicin was performed. DNA histograms were categorized into 4 types (I–IV) according to Auer [16] to estimate the grade of malignancy for diagnostic and prognostic purposes. Positions of stemline DNA aneuploidy peaks as well as the number of polyploidizing cells (>4.5C) were taken into account while analyzing DNA content profiles. The DNA content profiling data were compared with Miller-Payne histopathologic grades specifically designed for evaluation of therapeutic responses [17]. One of the therapy-resistant cases that showed extensive polyploidization was chosen for immunocytochemical staining to evaluate cell proliferation (Ki-67), invasiveness (CD44), and self-renewal factors typical for stem cells (SOX2 and NANOG).

Methods

Patients and clinico-pathologic information

The study involved 12 breast cancer patients (age range: 33–75), who underwent diagnostic procedures at the Latvian Oncology Centre of the Riga East University Hospital in 2014 and 2015. The tissue specimens were collected after the patients' informed consent was obtained in accordance with the regulations of the Committee of Medical Ethics of Latvia. The clinico-pathologic information about these patients, such as staging (ranged from I to IV), anatomic extent of disease according to TNM classification, overall grading (ranged from 1 to 3), proliferation status (based on scoring of Ki-67-positive cells in biopsies before treatment) and the status of ER, PR and HER2 receptors, was obtained from the aforementioned clinics. In the study group, 11 patients suffered from locally advanced breast cancer (predominantly Stage III disease). A case was diagnosed as triple-negative breast cancer (TNBC) if lack of ER and PR expression was accompanied by lack of HER2 expression as confirmed by commercial HercepTest (Dako, Glostrup, Denmark) showing HER2 levels at 0 or 1+. There were 7 cases diagnosed as TNBC. Such an excessive number of TNBC cases were collected intentionally because they were expected to be resistant to NAC. After completion of diagnostic procedures, the patients underwent 3–9 courses of NAC with standard doses of paclitaxel and doxorubicin followed by surgical removal of the affected tissue. NAC effects (therapeutic responses) were evaluated by Miller-Payne histopathologic grading system [17]. In brief, this grading system based on the comparison of tumor cellularity of the core biopsy (before NAC) with that of the resected tumor (after NAC) is presented as follows: grade 1 – no reduction in overall cellularity; grade 2 – a minor loss of tumor cells (<30%); grade 3 – moderate loss (30–90%); grade 4 – a significant loss (>90%); and grade 5 – no residual invasive cancer.

DNA image cytometry

The samples of diagnostic core biopsy or operation material (resected breast tissue specimens) were prepared on poly-L-lysine-coated microscope slides (Thermo Fisher Scientific, Waltham, MA, USA) and stained with a stoichiometric DNA dye toluidine blue (TB; Thermo Fisher Scientific) according to the published procedures [18, 19]. In brief, once samples had been fully air-dried on slides, they were fixed in acetone/ethanol mixture (1:1) for at least 30 min at 4 °C and dried again. Samples were treated with 5N HCI for 20 min at room temperature, washed in a distilled water (5×1 min), then stained with 0.05% TB in 50% McIlvain citrate-phosphate buffer (pH 4.0). Immediately after staining, samples were rapidly washed in distilled water (3 times by dipping) and promptly blotted with absorbing sheets of paper followed by dehydration in

butanol (2×3 min at 37°C). Samples were then immersed in xylene (2×3 min at room temperature) and embedded in DPX mounting medium (Sigma-Aldrich, St. Louis, MO, USA).

Digital images of at least 200 randomly selected and undistorted cell nuclei were collected with 100× objective magnification using Ergolux L03-10 microscope (Leitz, Germany) equipped with DXC-390P color video camera (Sony, Tokyo, Japan) calibrated in the green channel. DNA content per cell nucleus was measured as integral optical density using Image-Pro Plus 4.1 software (Media Cybernetics, Rockville, MD, USA). DNA content histograms were analyzed in accordance with the basic performance standards of diagnostic image cytometry [20]. DNA peaks were identified as aneuploid, if they deviated more than 10% from the normal diploid (2C in G_1) or tetraploid (4C in G_2) region (10% is the estimated integral error of the method). As a reference for locating normal 2C peaks, the nuclei of leukocytes persisting in a sample were analyzed as well. To classify breast cancer DNA content histograms, the approach proposed by Auer *et al.* [16] was used. For example, the histograms characterized by a single peak in 1.5–2.5C region (diploid and near-diploid region) were classified as type I, whereas the histograms characterized by a single peak in 3.5–4.5C region (tetraploid and near-tetraploid region) but sometimes supplemented with an additional peak in 1.5–2.5C region were classified as type II. In the type II histograms, the number of cells with ploidy of >4.5C together with the cells of ploidy ranged from 2.5C to 3.5C (predominantly near-triploid range) was less than 10%. The type III histograms represented highly proliferating cells (>5%) that scattered between normal or near-normal 2C and 4C peak positions. Less than 5% of cells had >4.5C. The type IV histograms were characterized by a large fraction of aneuploid cells (>5%) with increased and scattered DNA content significantly exceeding the normal 4C peak position (>4.5C). DNA histograms of types III and IV are indicative of the worst prognosis [16]. Ploidy-related parameters such as DNA index (DI) that characterizes aneuploidy and the percentages of cells exceeding 4.5C that characterize polyploidization were also determined. DI introduced by Barlogie et al. [21] was defined as the ratio of the modal DNA value of the abnormal cells in G_1 to the modal DNA value of the normal diploid cells in G_1.

Immunofluorescence staining

The imprints of surgically removed fresh tissue specimens were prepared on poly-L-lysine-coated microscopy slides (Thermo Fisher Scientific) followed by fixation and staining according to the published procedures [19]. Samples were fixed in methanol at −20°C for 7 min and dipped 10 times in ice-cold pure acetone. After fixation, samples were rinsed with Tris-buffered saline (TBS; pH 7.4) supplemented with 0.01% Tween 20 (washing solution abbreviated as TBST) 3×5 min and blocked in TBS supplemented with 0.05% Tween 20 and 1% BSA at room temperature. After blocking, samples were covered with TBS, 0.025% Tween 20, and 1% BSA, containing primary antibodies to Ki-67 (1:50, rabbit polyclonal, PA5-16785, Pierce), SOX2 (1:50, mouse monoclonal, MA1-014, Pierce), NANOG (1:50, mouse monoclonal, N3038, Sigma), and incubated in a humidified chamber overnight at 4°C. Then samples were washed with TBST 3×5 min at room temperature and incubated for 40 min at room temperature in the dark with the appropriate secondary antibodies diluted in TBST: goat anti-mouse IgG Alexa Fluor 488 (1:300, A31619, Invitrogen) or goat anti-rabbit IgG Alexa Fluor 594 (1:300, A31631, Invitrogen). Finally, samples were rinsed with TBST 3×5 min followed by rinsing with phosphate buffered saline (PBS; pH 7.3) 1×2 min. To clearly visualize cell nuclei, samples were counterstained with DAPI (Sigma-Aldrich) at concentration of 0.25 μg/ml for 2 min, rinsed with PBS, and embedded in the anti-fade reagent ProLong Gold (Invitrogen). As for staining of CD44, samples were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature followed by rinsing them with PBS containing 0.1% glycine 3×5 min. All subsequent steps were identical to those described above. Dilution of the primary antibody to CD44 (rabbit polyclonal, HPA005785, Sigma) was 1:50.

Results

Table 1 presents the clinico-pathologic information for each of 12 breast cancer cases supplemented by corresponding tumor DNA content analysis data that were obtained before and after NAC. The cases categorized in this study as near-triploid (~3C), whose DI=1.26–1.74 (n=7), were those that had at least one ~3C clone in spite of persisting the clones of other ploidy. The rest of the cases were defined as near-euploid, whose DI values fall into the category of <1.26 and/or >1.74 (n=5). The reason why we focused on the 'triploid group'

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Table 1. Clinico-pathological variables and image cytometry-based tumor DNA content analysis data of 12 breast cancer cases.

Patient No.	Age	Stage	Grade	ER %	PR %	Hercep Test (HER2)	Ki-67 %	Auer & DI before NAC	Auer & DI after NAC	> 4.5C % before NAC	> 4.5C % after NAC	Miller-Payne
3*	57	IIIB (T2N3M0)	3	0	0	0	86	IV (1.50)	IV (1.59)	22.5	23.8	1
5*	33	IIA (T2N0M0)	3	0	0	0	67	IV (1.00 /1.64)	III (1.00)	9.7	2.9	2
11	50	IIIA (T2N2M0)	3	0	0	3+	62	III (1.00)	III (1.00/1.73)	4.7	5.0	3
13	59	IIIA (T2N2M0)	2	65	0	3+	46	IV (1.48/1.70)	II (1.07)	6.6	0	2
15	75	IIIC (T2N3M0)	3	70	65	3+	72	IV (1.27/1.47)	IV (1.00/1.53)	9.1	13.6	2–3
17*	46	IIIC (T2N3M0)	3	0	0	0	78	IV (1.00/1.53)	IV (1.00/1.47)	11.8	19.5	1
26	57	IIIC (T4cN3M0)	2	0	0	2+	78	IV (1.13)	I (1.00)	4.9	0	4
27*	56	IIIA (T2N2M0)	3	0	0	0	92	III (1.00)	II (1.00)	1.3	0.9	2
29*	65	IIIB (T3N2M0)	3	0	0	1+	87	III (1.18)	III (1.14)	1.9	2.7	1
30*	38	IIIC (T2N3M0)	3	0	0	0	84	IV (1.07/1.67)	IV (1.00/1.64)	8.8	14.2	1–2
33*	34	IIIB (T3N2M0)	2	0	0	1+	24	IV (1.13/2.00/2.27)	II (1.20)	5.0	0.3	3
34	68	IIIC (T4cN3M0)	3	0	0	3+	62	IV (1.00/1.53/2.00)	II (1.41)	19.0	2.7	3

Denotations: Patient number is given as registered for this study; asterisk (*) – triple-negative breast cancer; NAC – neoadjuvant chemotherapy; Auer – DNA histogram typing that characterizes the extent of abnormality (types I–IV) according to Auer et al. [16]; DI – DNA index that shows the extent of aneuploidy; triploid or near-triploid DI values are underlined; Miller-Payne (grades 1–5) – tumor response to NAC according to Ogston et al. [17].

is that this category of patients compared with others was found to predominantly have highly malignant tumors leading to poor prognosis [14, 22–24]. In addition, the near-triploid cases can correlate with significant genomic gains (>5C) occurring presumably due to active proliferation of ~3C clones [22]. Although by examining diagnostic biopsies we did find an apparent increase in the number of >4.5C cells in near-triploid tumors compared with near-euploid ones (Fig. 1A), this finding was not clearly attributed to the enhanced proliferation of tumor cells, as evidenced by the results of counting of Ki-67-positive cells in both groups (Fig. 1B). Perhaps, much larger cohort of data is needed to delineate the role of tumor cell proliferation (particularly proliferation of ~3C clones) in gaining the numbers of >4.5C cells. Because an increase in the number of >4.5C cells assuming polyploidization is indicative of tumor aggressiveness and poor disease outcome, it seems reasonable to clarify whether NAC can eliminate them or considerably reduce their presence. Figure 2A shows that NAC completely eliminated >4.5C cells in 2 cases (13 and 26) and considerably reduced the percentage of such cells in 3 cases (5*, 33*, and 34), suggesting the apparent positive effect of NAC in 5 of 12 cases regardless of the ploidy group they belong to. However, among other 7 non-responsive cases (3*, 11, 15, 17*, 27*, 29*, and 30*), there were 3 cases (15, 17*, and 30*) that distinctly showed enhanced polyploidization, as evidenced by a significant increase in percentages of >4.5C cells, suggesting the negative (opposite) NAC effect. All those 5 aforementioned cases that were responsive to NAC showed the improved DNA content profiles evaluated according to Auer (Fig. 2B). Notably, the only one case (27*) of all non-responsive cases showed an improvement in DNA histogram from type III to type II. To demonstrate whether Auer DNA histogram typing data can correlate with Miller-Payne histopathologic scoring data, Figure 3 shows both of these parameters well interrelated. A distinct group of TNBC (3*, 5*, 17*, 29*, and 30*) is likely to be the most resistant to NAC, and all cases in this group except the case 29* belong to the near-triploid class. Although the case 27* showed an improved DNA histogram (Fig. 2B), this one had the low grade 2 (Miller-Payne scale, Fig. 3) confirming its resistance to NAC. As for the case 13 that was well responsive to NAC (Figs. 2A and 2B), this one, however, like the case 27*, had the low grade 2 (Miller-Payne scale, Fig. 3). Of all 12 cases, the best therapeutic effects were achieved with regard to the near-euploid case 26, which was not TNBC (Auer type I vs. Miller-Payne grade 4, Fig. 3). Regrettably, pCR was not achieved in all these cases because none of them had the highest Miller-Payne grade 5.

Discussion

Thus, the Auer types of DNA content profiles of post-NAC breast cancer cases were generally in agreement with corresponding Miller-Payne histopathologic grades, but quantification of >4.5C cells seems superior in terms of identi-

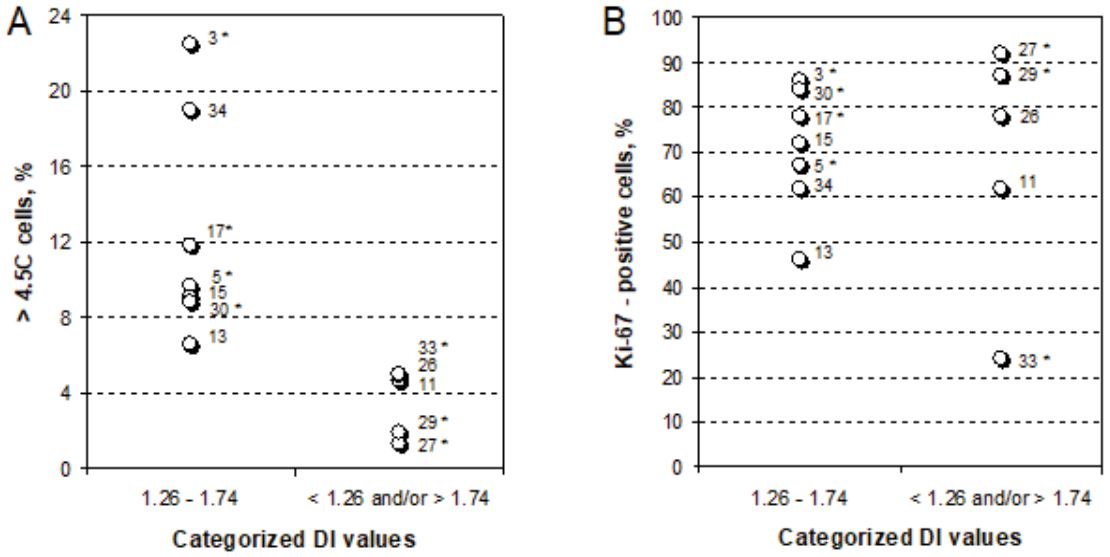

Fig. 1. Percentages of >4.5C cells (A) and Ki-67-positive cells (B) in near-triploid (DI values are ranged from 1.26 to 1.74) or near-euploid (DI values are <1.26 and/or >1.74) breast cancer cases before NAC. Asterisks (*) denote triple-negative breast cancer.

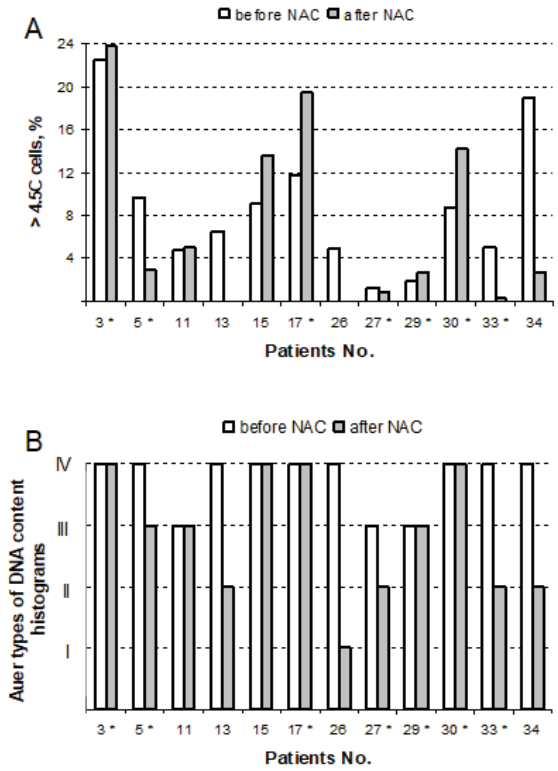

Fig. 2. Effects of NAC on percentages of >4.5C cells (A) and DNA content profiles (typed according to Auer) (B) in each of breast cancer cases. Asterisks (*) denote triple-negative breast cancer.

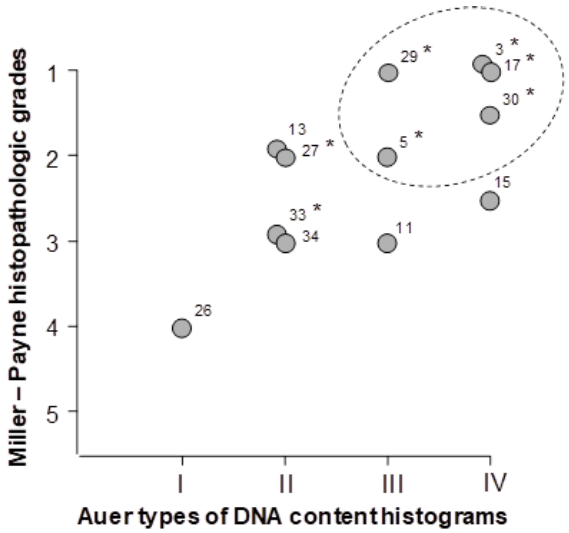

Fig. 3. Mapping of 12 breast cancer cases based on Auer typing of tumor DNA content histograms (after NAC) vs. Miller-Payne histopathologic scoring. Auer scale presents the types of histograms (I–IV), while Miller-Payne scale presents the histopathologic grades (1–5). Most resistant cases are encircled. Asterisks (*) denote triple-negative breast cancer.

fication of those cases that show the negative NAC effect (in this study those cases were 15, 17*, and 30*). Polyploidization that in some non-responsive cases was gained by NAC is likely to be attributed to ~3C clones. The most of non-responsive cases examined here are near-triploid TNBC, of which one case should especially be pointed out. This is the case 17*, which substantially differs from the rest of non-responsive cases in terms of spectacular post-NAC polyploidization of predominant 3C tumor cells resulting in the growth of the fraction of

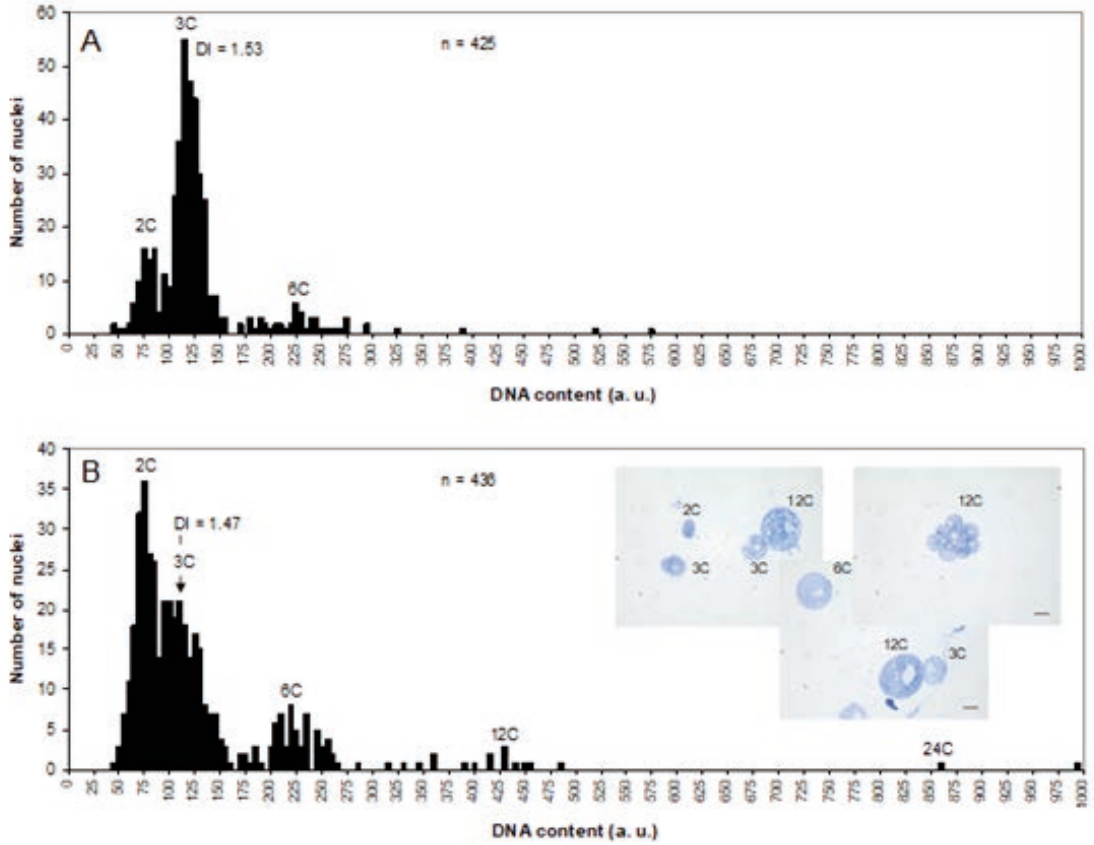

Fig. 4. DNA content histograms of a triple-negative case (patient 17*) before NAC (A) and after NAC accompanied with microphotographs of TB-stained nuclei (scale bars = 10 μm) (B).

6C cells accompanied by the appearance of additional fractions of 12C and 24C cells generated due to ploidy doublings (Fig. 4A vs. Fig. 4B). A significant increase of the fraction of 2C cells (Fig. 4B) may take place due to de-polyploidization [25]. Microscopic examination of the operation material obtained after NAC revealed the existence of multinucleated cells, although those ones were rarely seen (Fig. 4B). In this particular case (17*), like in some other non-responsive cases, cells with large nuclei were often observed as Ki-67-positive (Fig. 5A), supporting the assumption that these cells are capable of cycling. Among CD44-positive cells, cells with large nuclei were widely present as well (Fig. 5B), suggesting their invasive potential. Moreover, ~80% of 2C cells that may represent descendants of de-polyploidizing cells were CD44-positive. In basal-like breast cancers, of which 77% are TNBC [26], CD44-positive tumor cells that can potentially be associated with cancer stem cell phenotype are characteristic of highly aggressive tumors with enhanced invasiveness [27, 28]. Generation of drug-resistant polyploid tumor cells gaining mesenchymal characteristics with elevated expression of cancer stem cell markers (CD44 and CD133) has been reported [29]. In addition to the expression of Ki-67 and CD44, many cells with large or small nuclei (up to 75%) can express self-renewal factors characteristic for stem cells, such as SOX2 and NANOG (Figs. 5C-D and Figs. 5E-F, respectively). Notably, stress-induced polyploidization of tumor cells (including breast cancer cells) expressing self-renewal factors was also demonstrated *in vitro* in other studies by several groups of researchers, prompting us to suppose that the polyploid cells and their descendants released by de-polyploidization can possess stem cell features [30-33]. This type of stem cell-like gene expression signature is characteristic of poorly differentiated aggressive tumors including breast cancer often of basal-like subtype [34]. Interestingly, ionizing radiation may induce a breast cancer stem-cell phenotype $CD44^{+}/CD24^{-/low}$ in differentiated breast cancer cells with the involvement of polyploid cells that express OCT4, SOX2, NANOG, and KLF4, in a dose-dependent manner [32]. The number of examples demonstrating radio- or chemotherapy-induced either stem-like and/or therapy-resistant state in

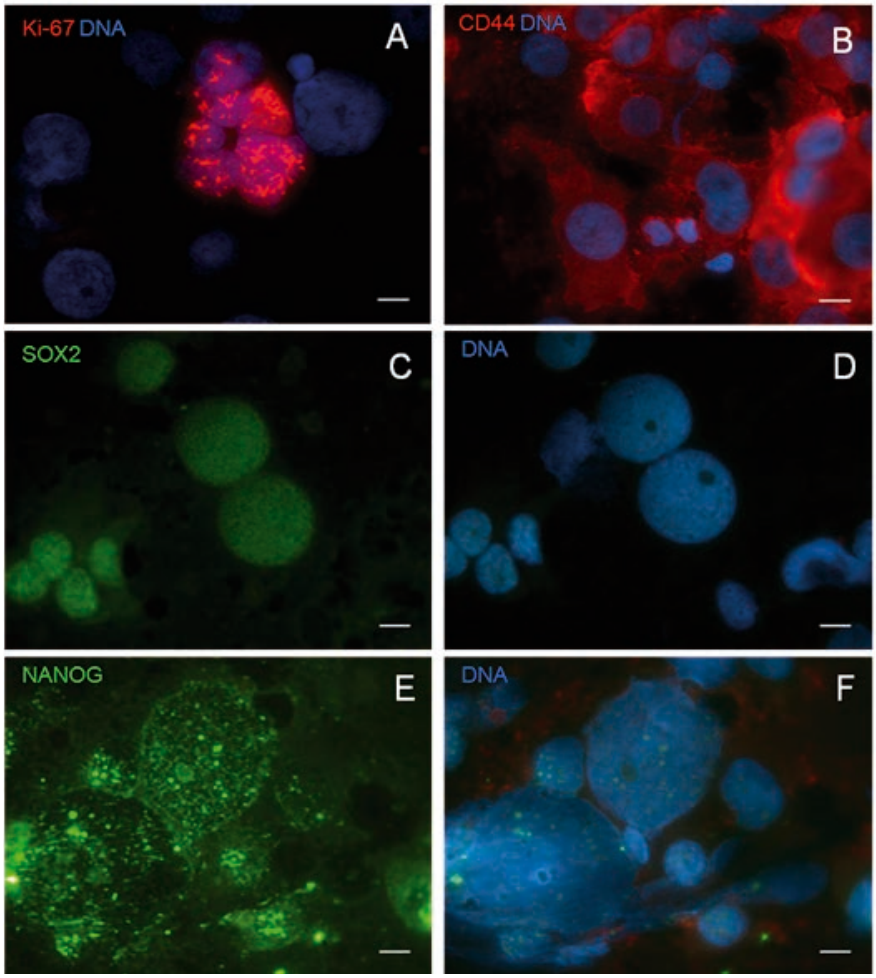

Fig. 5. Microphotographs of immunofluorescence-stained imprints of the surgical post-NAC material of patient 17* depicting cells positive for Ki-67, CD44, SOX2 and NANOG. (A) A group of large nuclei expressing Ki-67 in 15% of all Ki-67-positively stained nuclei. (B) Among CD44-positive cells (45%) there are many cells with large nuclei. (C, D) Among SOX2-positive cells (75%) there are many cells with large nuclei. (E, F) Among NANOG-positive cells (75%) there are many cells with large nuclei (in this image, two largest nuclei may have 24C). All immunofluorescence preparations were counterstained with DAPI for DNA. Scale bars = 10 μm.

tumor cells is growing [15, 35, 36]. A recent study of TNBC has shown that pre-existing subclones can be adaptively selected by NAC (adaptive resistance), followed by transcriptional reprogramming to evolve the resistant phenotypes [15].

Conclusions

DNA content profiling provides a facile, prompt, and accurate means of interpreting therapeutic responses in NAC-treated breast cancers. Because NAC effects for some breast cancers may be negative, the use of the further treatment strategy should be carefully considered. Therapy-resistant polyploid tumor cells possessing stem cell features can be induced *in vivo* as well, assuming that this process is not autonomous, but rather stipulated by the tumor microenvironment and intra-tumor heterogeneity. These polyploid cells are not quiescent and they manifest the invasiveness phenotype that together with stemness are also seen in descendants after de-polyploidization.

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Conflict of interest

The authors declare no conflict of interest.

ОЦІНКА ВІДПОВІДІ РАКУ МОЛОЧНОЇ ЗАЛОЗИ НА ПРОТИПУХЛИННУ ТЕРАПІЮ ЗА ДАНИМИ ДНК-ЦИТОМЕТРІЇ

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Вступ. *Розбіжності в інтерпретації відповіді раку молочної залози (РМЗ) на протипухлинну терапію все ще існують переважно через відсутність стандартизованих критеріїв оцінки та методів.*

Мета роботи. *Оцінка ефективності застосування профілювання вмісту ДНК у клітинах РМЗ до і після неоад'ювантної хіміотерапії (НХТ) для спрощення інтерпретації терапевтичного ефекту.*

Методи. *Препарати діагностичної біопсії та операційного матеріалу (тканини первинних пухлин, хірургічно видалених після НХТ), досліджували за допомогою ДНК-цитометрії. Поліплоїдію та анеуплоїдію у ДНК гістограмах оцінювали застосовуючи прогностичну типізацію Ауера. На тих же гістограмах визначали ДНК індекси (DI) клітинних клонів і відсотки поліплоїдизуючих клітин (>4.5C). Імунофлуоресцентне забарвлення застосовували для оцінки проліферації (Ki-67), інвазивності (CD44) та факторів самооновлення, притаманних стовбуровим клітинам (SOX2 та NANOG).*

Результати. *Профілювання вмісту ДНК 12 випадків РМЗ, з яких 7 були тричі-негативними, виявило ознаки нечутливості пухлини до НХТ у 7 випадках, з яких 5 були тричі-негативними. Серед нечутливих до НХТ випадків були 3 випадки, які показали посилену поліплоїдизацію, що свідчить про негативний ефект НХТ. Пара-триплоїдні (DI = 1.26-1.74) тричі-негативні випадки визначені як найбільш резистентні до НХТ. Проліферуючі пара-триплоїдні клітини можуть призводити до зростання кількості поліплоїдних клітин позитивних на Ki-67, CD44, SOX2 і NANOG.*

Висновки. *Дані ДНК-цитометрії забезпечують додатковою корисною інформацією для інтерпретації відповіді раку молочної залози РМЗ на протипухлинну терапію. Поліплоїдні пухлинні клітини з особливостями стовбурових клітин можуть бути індуковані НХТ. Оскільки в деяких випадках НХТ може викликати несприятливу відповідь пухлин, стратегію подальшої протипухлинної терапії необхідно ретельно переглянути.*

КЛЮЧОВІ СЛОВА: **рак молочної залози; поліплоїдні клітини; пара-триплоїдія; ДНК-цитометрія; терапевтичний ефект; стовбурові клітини раку.**

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PHARMACOKINETICS OF THE NEW CEREBROPETECTOR FERRUM BIS(CITRATO)GERMANATE AT THE STAGE OF ITS DISTRIBUTION TO THE ORGANS IN CLOSED HEAD INJURY

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Background. *Previous studies showed that new coordinate compound Cerebrogerm (ferrum bis(citrato) germanate) is a promising cerebroprotector.*

Objective *of the study is a comparative analysis of the central stage of the pharmacokinetics of Cerebrogerm as well as its distribution in vital organs in cases of closed head injury.*

Methods. *On the experimental original model of closed brain injury in rats the parameters of the central stage of Cerebrogerm pharmacokinetics: the distribution in brain, liver, kidneys, were studied.*

Results. *It is established that, in cases of closed head injury Cerebrogerm reaches maximum concentration first in the brain (in 3.75 h), then in the kidneys (in 3.92 h), and finally in the liver (in 4.17 h). In this case, the magnitude of the C_{max} of the coordinating compound of germanium that is being investigated in different biosubstrates of the rats with closed head injury may be presented in descending order as follows: brain (7.95 mg/L) > liver (6.22 mg/L) > kidneys (1.79 mg/L).*

Conclusions. *The compound Cerebrogerm studied easily gets through the blood-brain barrier and meets the present requirements for cerebroprotectors and antihypoxants. The attained results allow noting that in the early post-traumatic period of closed head injury, the blood circulation in the kidneys does not change and cannot modify the absorption-elimination processes of xenobiotics. It has been also established that Cerebrogerm is distributed faster in the examined organs in cases of closed head injury. The highest concentration of the drug is present in the brain and the smallest one – in the kidneys.*

KEY WORDS: **ferrum bis(citrato)germanat; closed head injury; pharmacokinetics; brain; liver; kidneys.**

Introduction

Closed head injury is characterized by a high and steadily increasing prevalence, which is especially marked during the period of hostilities. Consequences of brain injury have significant medical and social components that are related to treatment of patients and their rehabilitation. The variety of clinical manifes_ tations and the severity of closed head injury conditioned the need to develop a strategy for scientific approaches to its pathogenetic treatment [1, 2, 3].

In the medical practice, a very large arsenal of pharmacological methods of correction of closed head injury effects is currently used. At the same time, not all of them meet the current *requirements for their effectiveness and safety. Therefore, the search for and development of new cerebroprotective drugs with multidimensional pharmacodynamic effects and an acceptable pharmacokinetic profile are the dilemma. In this regard, the original compounds of germanium with different bioligands based on coordination bonds are particularly interesting [4, 5, 6].

The creation of new drugs is impossible without determination of pharmacokinetic parameters, which is a mandatory step in their preclinical studies and a prerequisite for further study of a potential drug in clinical practice. It is established that the clarification of the processes of getting drugs in the area of their pharmacodynamic effects (including cerebroprotective) has a great scientific and practical value in terms of developing a dosage regimen and optimizing rational pharmacotherapy in general.

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According to the results of screening studies on the model of traumatic brain damage in the original coordinating compounds of germanium with citric acid, it has been proved that the most active one is the complex compound ferrum bis(citrato)germanate of a conventional name Cerebrogerm [7].

The objective of the study was a comparative analysis of the central stage of the pharmacokinetics of ferrum bis(citrato)germanate, its distribution in vital organs in cases of normal as well as closed head injury.

Methods

The experiments were performed on 62 white mature inbred 160-220 g rats reared in the animal facility of the Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine. All procedures were performed according to the Ukrainian legislation, the European Convention on the protection of vertebrate animals used in experimental research and for other scientific purposes (1986) and local Ethics committee. The animals were on a standard diet and received food and water *ad libitum*.

The experimental model of the closed head injury is an acute pathological process that developed in rats kept in a fixed modified chamber after a single application of a force-metered and localization-oriented free-falling 45 g-load from the height of 80 cm onto the parietal skull [8].

Pharmacokinetic studies were conducted in accordance with the guidelines [9, 10] by determining the concentrations of Cerebrogerm by germanium in pathology-free tissues of rats and in those that received the studied coordinating compound for pharmacological correction of pathological changes occurring in the early post-traumatic period in cases of closed head injury. Cerebrogerm was determined in blood serum and in vital organs: cerebral cortex, liver and kidneys by the method [11].

Sampling of biomaterials in order to determine the pharmacokinetic profile of Cerebogerm was performed in dynamics: in 0.5; 2; 6; 12 and 24 hours from the time of single intraperitoneal rats injection of ferrum bis(citrato)germanate according to the previously established dosage regimen (126 mg/kg 70 min after closed head injury) [12].

For a maximum correct evaluation of the pharmacokinetics of a potential cerebroprotector, a number of parameters was defined. These parameters characterizing the process of distribution of a potential cerebroprotector in the body of a two-chamber kinetic model with absorption in health animals and with closed head injury comprise: time of reaching the maximum concentration (t_{max}, hours), maximum concentration (C_{max}, mg/l), period of half-distribution to the organ ($t_{1/2,a}$, hours), constant of the speed of direct mass transfer (K_{12}, h^{-1}), area under the pharmacokinetic curve (AUC, mg/L·min), period of the reverse mass transfer ($t_{1/2}$, hours), constant of the speed of reverse mass transfer (K_{21}, h^{-1}), average time in the organ (MRT, hours).

The attained results were statistically processed on a personal computer using the Student's t-distribution criteria and Mathematica V.5.0. Differences were considered statistically significant at $p<0.05$. The statistical analysis was conducted at a 95% confidence level.

Results

The results of the pharmacokinetic study which characterize the process of distribution in organs are presented in Fig. 1 and 2 in the form of kinetic curves that indicate Cerebrogerm distribution into the brain, liver and kidneys.

Pharmacokinetic parameters that characterize the process of distribution of Cerebrogerm in vital organs of healthy animals (control group) and in those with closed head injury are presented in Table 1.

It is established that, in cases of closed head injury Cerebrogerm reaches maximum concentration first in the brain (in 3.75 h), then in the kidneys (in 3.92 h), and finally in the liver (in 4.17 h). In this case, the magnitude of the C_{max} of the coordinating compound of germanium that is being investigated in different biosubstrates of the rats with closed head injury may be presented in descending order as follows: brain (7.95 mg/L)> liver (6.22 mg/L)> kidneys (1.79 mg/L).

At the same time, Cerebrogerm is identified in maximum concentration in the brain of healthy animals in 5.26 h, in the kidneys in 5.10 h and in the liver only in 6.20 h, with the presumable difference from the experimental group in relation to tmax in all biosubstrates. Simultaneously, under normal conditions the organs of rats can be placed according to the size of the Cmax of Cerebrogerm in descending order as follows: brain (7.09 mg/L)> liver (5.71 mg/L)> kidneys (1.68 mg/L).

The distribution (its intensity and completeness) of Cerebrogerm to the peripheral

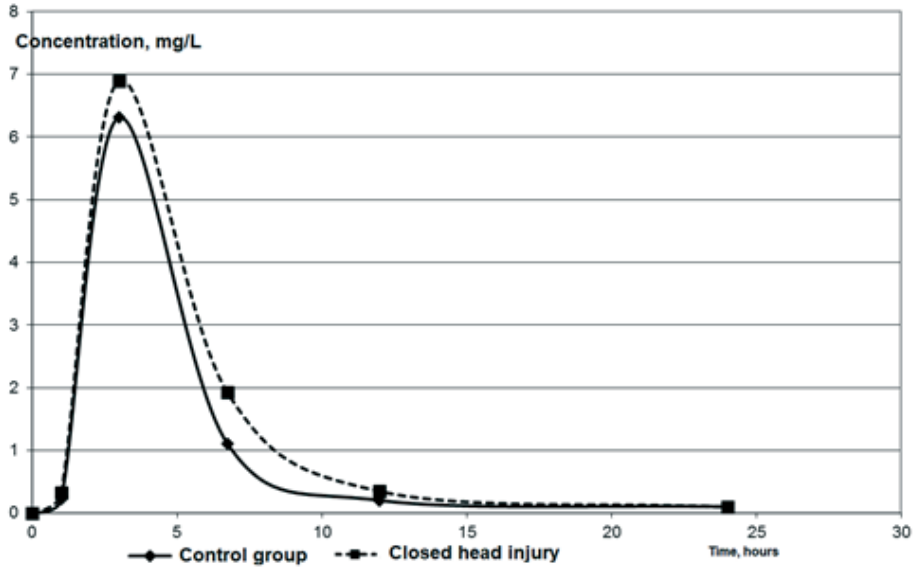

Fig. 1. Pharmacokinetic curves showing the concentration of Cerebrogerm in the brain of rats of the control group as well as in cases of closed head injury after a single intravenous injection at the dose of 126 mg/kg.

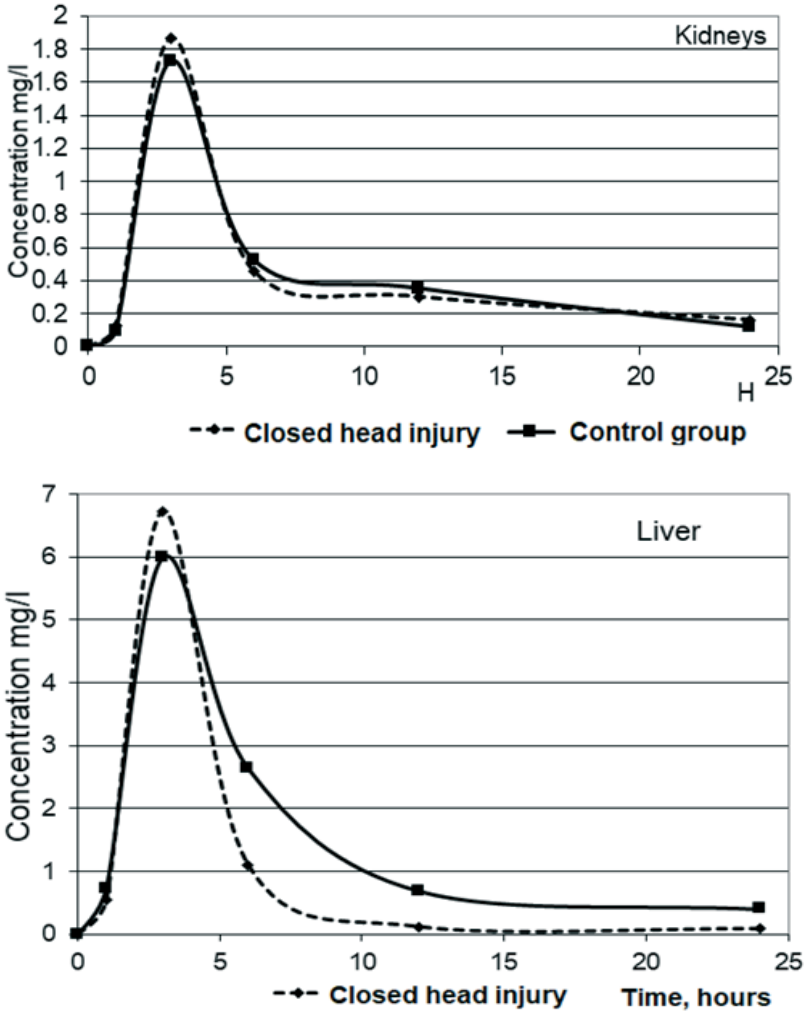

Fig. 2. Pharmacokinetic curves of the concentration of Cerebrogerm in the kidneys and liver of the control group rats as well as in those with closed head injury after a single intravenous injection at the dose of 126 mg/kg.

Table 1. Pharmacokinetic parameters of Cerebrogerm in the process of its distribution to the peripheral chambers of the kinetic model of healthy rats (the control group) and those with closed head injury after a single intravenous injection at the dose of 126 mg/kg (n = 6).

Pharmacokinetic parameter	Groups of animals					
	Brain		Liver		Kidneys	
	Control group	Closed Head Injury	Control group	Closed Head Injury	Control group	Closed Head Injury
Time of reaching maximum concentration (tmax, year)	5.26±0.32	3.75±0.27*	6.20±0.47	4.17±0.12**	5.10±0.11	3.92±1.34*
Maximum concentration (Cmax, mg/L)	7.09±0.15	7.95±0.10**	5.71±0.17	6.22±0.14	1.68±0.05	1.79±0.02
Period of half-distribution to the organ, ($t_{1/2, a}$, h)	1.99±0.05	1.08±0.07***	1.59±0.06	1.25±0.02**	4.79±0.01	2.68±0.04***
Constant of the speed of direct mass transfer, (K_{12}, h^{-1})	0.35±0.01	0.64±0.001***	0.44±0.001	0.55±0.001***	0.14±0.001	0.26±0.001***
Area under the pharmacokinetic curve (AUC, mg/L·min)	424367.30 ± 2744.02	511245.70 ± 1682.77***	322145.40 ± 1567.37	224394.90 ± 784.12***	137029.20 ± 768.50	127140.0 ± 224.17***
Period of reverse mass transfer, ($t_{1/2}$, h)	2.05±0.04	3.07±0.16***	1.79±0.09	1.24±0.07**	2.37±0.22	2.45±0.08
Constant of the speed of reverse mass transfer, (K_{21}, h^{-1})	0.34±0.01	0.23***±0.01	0.39±0.01	0.56±0.02***	0.29±0.01	0.28±0.02
Average time in the organ (MRT, h)	24.7±0.73	24.5±2.17	30.1±1.97	21.7±1.46*	31.9±2.72	30.7±1.47

Notes: * – p <0.05 compare to the healthy animals; ** – p <0.01 compare to the healthy animals; *** – p <0.001 compare to the healthy animals.

chamber is present in the half-distribution period ($t_{1/2, a,}$ h) from the central chamber to the studied organs and the constant of the speed of direct mass transfer (K_{12}). It has been established that in cases of closed head injury (research), the coordinating compound of germanium reaches the brain the most fast, the liver slightly slower, and the kidneys the slowest, as evidenced by the parameters of $t_{1/2, a}$, which denotes the time of reaching the levels of the compound which is equal to 50% of equilibrium concentrations between blood and other tissues.

Taking into account that the parameter K_{12} defines the relation $K_{12} = 0.693/t_{1/2',a}$, that is the parameter is inversely proportional to the half-distribution period in the organs, then the revealed changes of K_{12} also confirm the above-described character of the distribution of the compound in the organs. It is established that Cerebrogerm in both groups of animals equally enters the kidneys slowly, and the brain – the most intensively.

Such a parameter as the area under the pharmacokinetic curve (AUC) is particularly informative. It is proportional to the amount of the compound present in blood. It was established that under normal conditions the highest concentration of the drug was revealed in the brain and the smallest – in the kidneys. The following sequence follows the AUC indices: brain > liver > kidneys (see Table 1).

A comparative estimation of the AUC of pathology-free animals and those of the experimental group indicates a significant difference between the concentrations of Cerebrogerm in liver. It is established that the potential cerebroprotector is revealed in the liver under normal conditions by 43.6% more than in cases of closed head injury.

It should be noted that Cerebrogerm enters the brain ($t_{1/2, a}$) very fast, but eliminates ($t_{1/2}$) slowly in both compared groups of animals. It is significant that Cerebrogerm is almost identically eliminated from the kidneys of the rats with or without traumatic brain damage ($p > 0.05$).

Similarly, the organs are classified according to the constant of the speed of reverse mass transfer. As presented in Table 1, Cerebrogerm eliminates from the liver of the animals of the experimental group the fastest and from the brain the slowest.

Among the pharmacokinetic parameters studied during the preclinical stage, the average time the compound stays in the body has high informational value since the implementation of the pharmacological effect of a drug directly depends on its time in the whole body as well as in a particular organ or tissue. The results presented in Table 1 evidence that in the pathology-free animals the studied organs are classified according to the MRT decrease as follows: kidneys (31.9 h)> liver (30.1 h)> brain (24.7 h).

In cases of traumatic brain damage, Cerebrogerm is eliminated by 27.9% faster (21.7 hours) from the liver with no significant differences in MRT of the brain and kidneys ($p < 0.05$).

Discussion

The attained data, which characterizes the speed of Cerebrogerm being delivered from blood to various animal organs (see Table 1), indicates that coordination compound of germanium enters the peripheral chambers of the biokinetic model in different speed and amount both under normal and closed head injury conditions. It is worth noting that Cerebrogerm passes through the histo-hematic barriers of the studied organs in different speed.

The defined pharmacokinetic parameters of Cerebrogerm, characterizing the process of getting from the peripheral chambers of the kinetic model into the blood under normal conditions as well as in cases of closed head injury, require more detailed analysis and discussion. Particular attention should be paid to the period of reverse mass transfer ($t_{1/2}$), that is the time the compound returns to the central circulation of the peripheral chamber (organ). According to this indicator, the pathology-free organs of the animals take the order of: liver > kidneys > brain.

The revealed character of the distribution of Cerebrogerm in cases of closed head injury indicates, in our opinion, the direction of its action in terms of cerebroprotective effect. Under normal conditions in the animals Cerebrogerm is first rewealed in liver, and then in the brain and kidneys.

In this case, a 32.4% ($P < 0.001$) decrease was defined in the rate of the reverse mass transfer constant of Cerebrogerm from the brain into the blood in cases of closed head injury caused by post-traumatic exudation of the brain tissue which was subjected to mechanical damage.

Consequently, cerebral edema which occurs in cases of closed head injury makes the coordination of germanium stay in the brain longer that eventually causes a decrease in the

speed of Cerebrogerm transition from the brain into the bloodstream.

This data reaffirms our hypothesis about cerebral edema, which impedes the transition of Cerebrogerm from the brain into the blood, as evidenced by ($p<0.001$) increase of the AUC indices by 20.5% in cases of closed head injury compare to normal conditions.

This experimentally proved fact can indirectly show the detoxification function of the liver being saved in terms of its direct involvement in the processes of elimination of Cerebrogerm as a xenobiotic.

Thus, it should be noted that in the early post-traumatic period of closed head injury, blood circulation in kidneys does not change to the extent that might substantially modify the absorption-elimination processes of xenobiotics. This is quite appropriate in view of the fact that kidneys have rich blood supply.

Thus, the results of the complex preclinical studies prove that the defined pharmacokinetic profile of the distribution of Cerebrogerm in cases of closed mechanical brain trauma differs from that under the normal conditions. It has been experimentally proved that Cerebrogerm enters the examined organs in cases of closed head injury faster than it does without it. It should be noted that the highest concentration of the drug, as evidenced by the area under the pharmacokinetic curve, occurs in the brain of the animals with closed head injury and the smallest – in the kidneys.

This should be considered as a key pharmacokinetic parameter that clearly proves the ability of Cerebrogerm to get through the blood-brain barrier. The attained data also proves that the coordination compound of germanium corresponds precisely to the current requirements for cerebroprotectors and antihypoxants. This kind of drugs is capable to implement a number of pharmacodynamic effects through the accumulation in the brain, as in the central biophase. These effects aim at organ protection, mainly the protection of cerebral neurons.

Conclusions

Determination of the pharmacokinetic parameters of the potential cerebroprotector ferrum bis(citrato)germanate at the stage of its distribution in the body in cases of traumatic brain damage indicates a significant modification of the kinetic profile of the compound compared to the normal conditions. A number of parameters that characterize the process of Cerebrogerm's distribution from the central chamber to the peripheral ($t_{1/2,a}$, K_{12}, K_{21}, AUC) substantially ($P<0.05$ - 0.001) undergoes a 'traumatic' correction. According to the parameters characterizing the pharmacokinetic spectrum of the distribution of Cerebrogerm into organs, it can be represented in the following order: brain > liver > kidneys.

The studied compound Cerebrogerm easily gets through the blood-brain barrier and meets the present requirements for cerebroprotectors and antihypoxants. The attained results allow noting that in the early post-traumatic period of closed head injury, the blood circulation in the kidneys does not change and cannot modify the absorption-elimination processes of xenobiotics. It has been also established that Cerebrogerm is distributed faster in the examined organs in cases of closed head injury. The highest concentration of the drug is present in the brain and the smallest one – in the kidneys.

Conflict of interest

The authors declare no conflict of interest.

ФАРМАКОКІНЕТИКА НОВОГО ЦЕРЕБРОПРОТЕКТОРА ФЕРУМ БІС(ЦИТРАТО) ГЕРМАНАТУ НА ЕТАПІ ЙОГО РОЗПОДІЛУ В ОРГАНИ НА ТЛІ ЗАКРИТОЇ ЧЕРЕПНО-МОЗКОВОЇ ТРАВМИ

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Вступ. *Попередні скринінгові фармако- та токсикометричні дослідження нової координаційної сполуки Цереброгерм (ферум біс(цитрато)германат) показали, що це перспективний потенційний засіб церебропротекції.*

Мета дослідження - *провести порівняльний аналіз центрального етапу фармакокінетики Цереброгерму – розподілу у життєвоважливі органи: головний мозок, печінка, нирки, в нормі та при ЗЧМТ.*

Матеріали. *На оригінальній моделі ЗЧМТ визначали низку фармакокінетичних параметрів ферум біс(цитрато)германату що всебічно характеризують процес розподілу в периферичні камери моделі в нормі та за умов ЗЧМТ.*

Результати. *Показано, що при ЗЧМТ максимум концентрації Цереброгерму досягається спочатку у мозку (після 3,75 год), потім у нирках (після 3,92 год) та у печінці (після 4,17 год). В той же час, за величиною C_{max} Цереброгерму органи щурів контрольної групи можна розташувати у порядку зменшення наступним чином: головний мозок (7,09 мг/л) > печінка (5,71 мг/л)> нирки (1,68 мг/л). Величина C_{max} координаційної сполуки германію у щурів із ЗЧМТ виглядає так: головний мозок (7,95 мг/л) > печінка (6,22 мг/л)> нирки (1,79 мг/л).*

Висновки. *Отримані дані дозволяють дійти висновку, що досліджувана сполука доволі легко проникає через гематоенцефалічний бар'єр і відповідає сучасним вимогам до церебропротекторів та антигіпоксантів. Отримані результати дозволяють зазначити, що у ранньому постравматичному періоді ЗЧМТ стан кровообігу у нирках не змінюється у такій мірі, щоби суттєво модифікувати абсорбційно-елімінаційні процеси ксенобіотика. Доведено також, що за умов ЗЧМТ Цереброгерм надходить до органів, що досліджувались, більш швидко, ніж у здорових щурів. Найбільша концентрація препарату, про яку судили за показником площі під фармакокінетичною кривою, знаходиться у головному мозку, а найменша - в нирках травмованих тварин.*

КЛЮЧОВІ СЛОВА: **ферум біс(цитрато)германат; закрита черепно-мозкова травма; фармакокінетика; головний мозок; печінка; нирки.**

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EFFICACY OF GRANULOCYTE COLONY-STIMULATING FACTOR AND ENTEROSORPTION IN MELPHALAN-INDUCED BONE MARROW SUPPRESSION IN GUERIN CARCINOMA GRAFTED RATS

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Background. *Side effects of antineoplastic agents (especially leukopenia and neutropenia) could be the main limiting factors for efficient treatment.*

Objective. *The research is aimed at the study of myeloprotective capability of biosimilars of granulocyte colony stimulating factor (G-CSF) and granular carbon oral adsorbent C2 in melphalan-induced bone marrow suppression in Guerin carcinoma-grafted rats.*

Methods. *Melphalan at the dose of 5.5 mg/kg was used to promote bone marrow suppression in the Guerin carcinoma grafted rats. To fight myelosuppression, we used filgrastim and its analogue, designed and produced by IEPOR, a recombinant granulocyte colony-stimulating factor (r-GCSF). Carbon granulated enterosorbent C2 was used for enteral sorption therapy (bulk density γ=0.18 g/cm^3, diameter of granules 0.15-0.25 mm, BET pore surface – 2162 m^2/g). All rats were sacrificed on the 17th day after carcinoma cells inoculation or on the 8th day after Melphalan injection.*

Results. *Alkylating cytostatic agent caused severe leukopenia (by 95.7%), neutropenia (by 73.9%), and thrombocytopenia (by 84.9%) in the experimental rats. Mortality rate was 57%. Filgrastim and enterosorption with carbon oral adsorbent C2 increased the studied indices, but the most prominent results were observed when combination of both factors was used. Studied means did not affect the anti-tumor efficacy of Melphalan alone and in combination.*

Conclusions. *Our results are perspective for further investigation of the efficacy of the combination of carbon oral adsorbents and hematopoietic cytokines in cases of ameliorate anti-cancer chemotherapy side effects, and its implementation into clinics.*

KEY WORDS: **melphalan; Guerin carcinoma; rats; granulocyte colony stimulating factor; enterosorption.**

Introduction

Conventional dose-dense and dose-intense chemotherapy with radiation therapy and surgery promote significantly to the treatment of malignancies. Unfortunately, the side effects of antineoplastic agents are the main limiting factors for their efficiency [1–3]. The cells and tissues with high speed of division are the most sensitive. Bone marrow suppression, damage of gastrointestinal mucosa, gonadal toxicity, loss hair, dysfunctions and/or structural changes of kidney and liver are typical side effects of tumoricidal chemotherapy [2,4,5]. The incidence rates of myelotoxicity varies from 30 up to 60% [6]. The mortality rates due to febrile neutropenia among the patients undergoing chemotherapy are around 5-11% in adults and 2-6% in children [7,8], and up to 20% in case of comorbidity [6]. So, amelioration of the negative effects and saving of the tumoricidal activity of chemotherapy are topical issues of contemporary oncology. Only complete courses of treatment may guarantee survival [7,9].

Granulocyte colony-stimulating factor (G-CSF) and a granulocyte-macrophage colony-stimulating factor (GM-CSF) are an irreplaceable part of supportive care in oncology and are used to reduce the incidence of severe leukopenia [10]. It has been proved in the cancer patients receiving multiple cycles of chemotherapy [11].

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Endogenous intoxication is another component that makes the side effects of chemotherapy more severe. These syndrome compounds are the products of tumor metabolism, and the consequence of treatment (surgery, chemotherapy cause the damage of tumor tissues and increase in the quantity of toxic metabolites as well as its derivatives) [12]. Sorption Detoxification is a common and well-known method for reducing toxic effects of chemotherapy [13–15]. Some of the main types, which are widely used in medicine today, are hemoperfusion (when blood is filtered through the column with activated carbon), enterosorption – enteral use of oral adsorbents of different types, and application-sorption therapy – use of carbon dressing for healing of burns and wounds.

Our previous experiments proved a significant bone marrow protection in melphalan-induced myelosuppression in the healthy rats [16,17]. But a topical issue of any additive supportive therapy in oncology is the impact on a tumor growth, not only the amelioration of anti-cancer chemotherapy side effects. So, the objective of our investigation is the study of myeloprotective capability of biosimilars of granulocyte colony stimulating factor (G-CSF) and enteral sorption therapy with carbon oral adsorbent C2 in Melphalan-induced bone marrow suppression in the Guerin carcinoma grafted rats.

Methods

Melphalan (Alkeran, GlaxoSmithKline, UK) was used to promote the bone marrow suppression. Carbon granulated enterosorbent C2 (produced by R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, IEPOR) was used for enteral sorption therapy. The parameters of enterosorbent C2 are: bulk density γ=0.18 g/cm^3, diameter of granules 0.15–0.25 mm, BET pore surface – 2162 m^2/g. Filgrastim (Neupogen, Hoffman La Roche) and G-CSF-analogue of IEPOR production – recombinant granulocyte colony-stimulating factor (r-GCSF) were used to increase the white blood cell (WBC) count. r-GCSF was designed within the State Grant No. 487/2011 from 29.09.2011 "Development of technology for synthesis of human recombinant granulocyte colony stimulating factor and medication on its basis".

The experiments were performed using mature Wistar female rats, 200±20 g of body mass, which were got from the vivarium of IEPOR (Kyiv, Ukraine). Guerin carcinoma cells were taken from the Bank of Cell Lines from Human and Animal Tissues of the abovementioned institute. All procedures were performed according to the rules and requirements of European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and a local Ethic Committee of IEPOR. The tumors were implanted by subcutaneous injection (dorsally into the left flank) of 2.2×10^6 Guerin carcinoma cells suspended in 0.5 ml of sterile physiological solution. All animals were randomly divided into 6 groups (n=7). The 1st group was a tumor control group. All rats of groups 2-6 were administered with Melphalan: the 3rd group involved the rats, which were administered with filgrastim injections (Melphalan + filgrastim) in addition to the cytostatic agent; the 4th – enteral sorption therapy with C2 (Melphalan + C2), the 5th group – the rats, which were administered with both agents (Melphalan + filgrastim + C2), and the 6th group – the animals, which received r-CSF and carbon oral adsorbents C2 (Melphalan + r-CSF + C2).

On the 10th day after Guerin carcinoma grafting, Melphalan was injected intravenously (into the tail vein) one time at the dose of 5.5 mg/kg to the rats of groups 2-6. Beginning from the following day and for four next days, filgrastim or r-CSF was injected subcutaneously at the dose of 50 mcg/kg. A suspension of carbon enterosorbent (C2 dosage of 5 ml/kg of animals' body weight, or 900 mg of dry mass of enterosorbent) in appropriate quantity of distilled water was introduced via the tube into rat stomach during 3 days before the Melphalan injection and during 7 days after it (once a day).

The rats' mortality rate and dynamics of tumor growth was studied as well. The rats were weighted, and blood was taken from the heart under Ketamine hydrochloride general anesthesia on the 8th day after Melphalan injection (the 17th day after Guerin carcinoma cells inoculation). Automatic hematology analyzer Particle Counter E120 (Erma Inc., Japan) was used for evaluation of complete blood cell count.

Statistical analysis. Since data were not normally distributed in all groups (Shapiro-Wilk Normality Test), non-parametric tests (Mann-Whitney U-test and one-way ANOVA test) were used in data analysis (statistical significance at $p<0.05$). The data were expressed as the mean± standard error of the mean (M±SE). All statistical calculations were performed using the Origin 7.5 Software (OriginLab Corporation, USA).

Results

All rats survived in the tumor control group on the 17th day after Guerin carcinoma cells inoculation, while Melphalan injection caused the death of 4 rats (the 2nd group). In the group of rats, which received carbon oral adsorbent, 2 rats died. In all other groups one rat died in each group till the end of experiment (Figure 1). So, we evidenced the mortality rate of 57.1 % in the Melphalan group, while the combination of both agents of correction (G-CSF and carbon oral adsorbent) reduced this number to 14.3 %.

It was established that a single injection of cytostatic agent Melphalan caused significant leukopenia from (13.9±1.9) to (0.6±0.1)×10^9/L (Figure 2). The decreasing of WBC count by 95.7% explained a high mortality rate in the Melphalan group (the 2nd group). Course of Filgrastim promoted 2.3-fold increase of WBC count (or by 133.3%) compare to the Melphalan group. Enteral sorption therapy also increased this index in 1.5 times (or by 50.0%). The use of combination of both G-CSF drugs and enterosorbent had the tendency to be more effective compare to the administration of each drug alone. The WBC count increased in 2.8 times (or by 183.3%) in the Melphalan+filgrastim+C2 group and in 2.7 times (or by 166.7%) in the Melphalan+r-GCSF+C2 group compare to the Melphalan group.

As for the WBC formula, the results of the experiment are presented in Figure 3.

Single Melphalan injection at the dose of 5.5 mg/kg^{-1} significantly decreased the granulocytes percentage in the peripheral blood by 73.9%, monocytes were absent at all. The

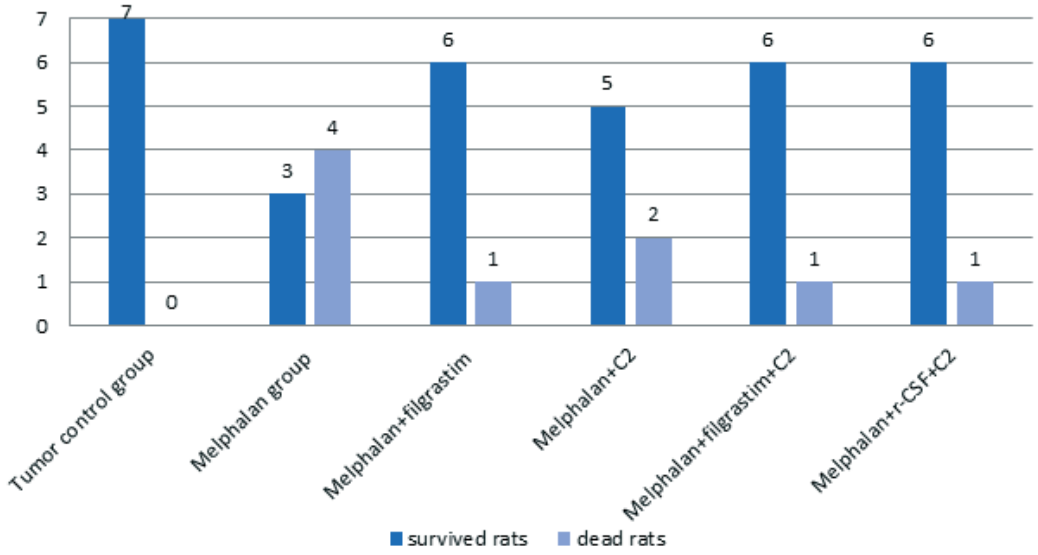

Figure 1. Survival and mortality rates among the Guerin carcinoma grafted rats, which received Melphalan, biosimilars of G-CSF and enterosorbent C2 on the 17th day after tumor cells inoculation.

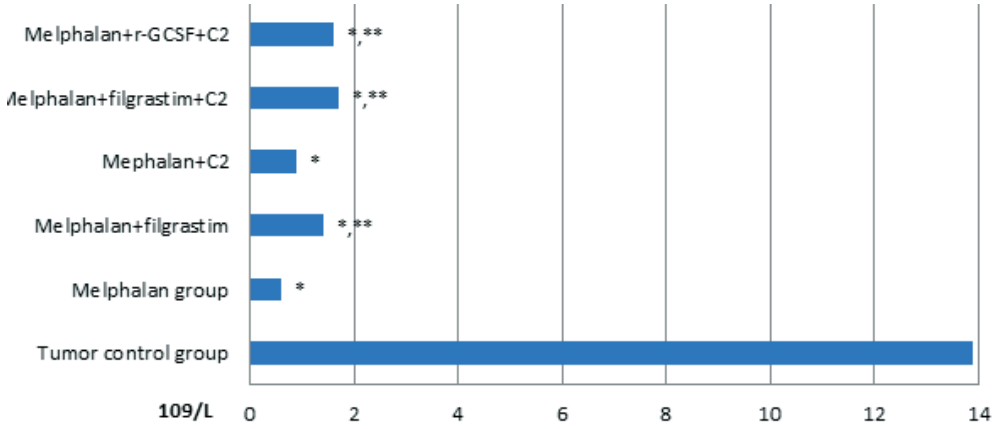

Figure 2. White blood cell (WBC) count in the Guerin carcinoma grafted rats, which received Melphalan, biosimilars of G-CSF and enterosorbent C2 on the 17th day after tumor cells inoculation.
Notes: * – p<0.05 compare to the tumor control group; ** – p<0.05 compare to the Melphalan group.

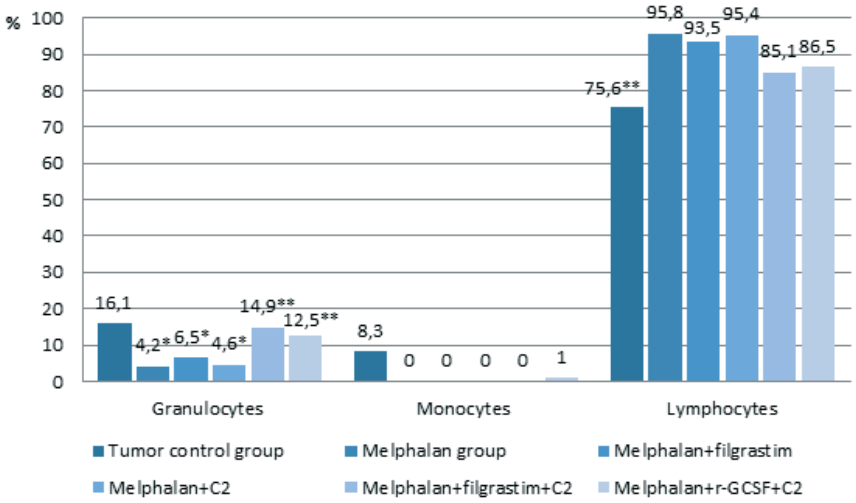

Figure 3. White blood cell (WBC) formula of the peripheral blood in Guerin carcinoma grafted rats, which received Melphalan, biosimilars of G-CSF and enterosorbent C2 on the 17th day after tumor cells inoculation. Notes: * – p < 0.05 compare to the tumor control group; ** – p < 0.05 compare to the Melphalan group.

increased number of lymphocytes in the peripheral blood was evidenced that can be explained by critically low WBC count and violation of cells composition. The administration of filgrastim and carbon oral adsorbent C2 alone did not influence significantly; a slight tendency of increase of the indices was evidenced. The combination of both factors led to normalization of granulocytes percentage in the peripheral blood, while the number of leukocytes was still quite low (Figure 2). So, granulocytes number increased by 254.8% (in 3.6 times) in the the Melphalan+filgrastim+C2 group and by 197.6% (approximately in 3 times) in the Melphalan+r-GCSF+C2 group compare to the Melphalan group.

As for the red blood cell (RBC) count and hemoglobin, no significant impact of any drug used in the experiment was evidenced. But a 6.6-fold decrease of platelets count was observed in the rats (or by 84.9%), which were administered with Melphalan compare to the tumor control group (Table 1).

The same tendency as for the granulocytes percentage was evidenced concerning the platelets count: only the tendency for amelioration of low platelets level. The combination of both drugs: officinal filgrastim or specially designed analogue r-GCSF together with carbon oral adsorbent C2, caused significant increase of thrombocytes number in peripheral blood. Thus, platelets count increased in 4.2 times (or by 319.6%) in the Melphalan+filgrastim+C2 group, and approximately in 3 times (or by 198%) in the Melphalan+r-GCSF+C2 group compare to the 2nd Melphalan group.

The tumor growth dynamics in case of the administration of alkylating cytostatic agent and G-CSF analogues as well as enterosorption was a significant aim of the study. Melphalan was injected on the 10th day after Guerin carcinoma cells inoculation, when a tumor was formed (Table 2). On the 17th day of the experiment a significant slow of tumor growth compare to the tumor control group was evidenced. There was no negative effect of

Table 1. RBC and platelets count as well as hemoglobin level in peripheral blood in the Guerin-carcinoma grafted rats in case of Melpahaln, G-CSF-analogues and enteral sorption therapy, M±SE

Indices / Group	RBC count, $\times 10^{12}$/L	Platelets count, $\times 10^{9}$/L	Hb, g/L
Tumor control group, (n=7)	7.73±0.51	325.6±35.9**	152.0±8.0
Melphalan group, (n=3)	8.70±2.65	49.0±11.9*	130.0±30.0
Melphalan + filgrastim group, (n=6)	10.57±1.19	74.0±9.5*	211.0±25.0
Melphalan + C2 group, (n=5)	8.06±0.68	60.0±8.6*	153.0±15.0
Melphalan + filgrastim + C2 group, (n=6)	10.21±1.00	205.6±86.2**	188.0±18.0
Melphalan + r-GCSF + C2 group, (n=6)	7.91±0.81	146.0±36.8*,**	146.0±17.0

Notes: * – p<0.05 compare to the tumor control group; ** – p<0.05 compare to the Melphalan group.

Table 2. Dynamics of the Guerin carcinoma growth in the experimental groups (volume, cm^3, M±SE, n=7)

Group	Day after tumor's inoculation	
	10th day	17th day
Tumor control group	3.7±0.2	11.3±0.2
Melphalan group	3.8±0.3	6.6±0.2 *
Melphalan + filgrastim group	3.7±0.2	6.4±0.3 *
Melphalan + C2 group	3.5±0.2	6.1±0.2 *
Melphalan + filgrastim + C2 group	3.8±0.3	6.0±0.4 *
Melphalan + r-GCSF + C2 group	3.7±0.4	6.0±0.3 *

Notes: * – $p<0.05$ compare to the tumor control group.

G-CSF drugs or enterosorbent C2 on the anti-cancer action of Melphalan. On the 17th day the tumors sizes decreased by 41.6 % in the Melphalan group, by 43.4 % in the Melphalan+ filgrastim group, by 46.0 % in the Melphalan+C2 group, and by 46.9 % in the groups of rats, which received combination of enterosorption and hematostimulating cytokines. We assessed the tumors size in all groups of rats.

Discussion

Typical side effects of anti-cancer chemotherapy, and leukopenia as the most common among them, are tightly related to the clinical outcomes [1, 18]. More and more attention is paid to this issue, especially after the success of modern techniques such as a stem cell transplantation and cytokines treatment to restore hematopoietic functions. Different chemotherapy regimens are classified to develop a high risk (more than 20%), an intermediate risk (from10 to 20%) or a low risk (less than 10%) of febrile neutropenia [6]. All of them require administration of different doses of hematopoietic cytokines to prevent and treat complication of tumoricidal therapy. G-CSF influences cellular proliferation, differentiation, maturation, and lineage commitment in the bone marrow not only of neutrophils but of short-term hematopoietic stem cells, colony forming units granulocyte erythroid macrophage megakaryocyte, colony-forming units granulocyte macrophage and colony forming units granulocyte as well [19]. The American Society of Clinical Oncology (ASCO) recommends primary prophylaxis with G-CSF or GM-CSF for the expected incidence of neutropenia of ≥40% [20]. Also, pegfilgrastim (sustained duration form of filgrastim, G-CSF) is recommended for administration for at least 1 day after chemotherapy [21].

G-CSF drugs are mostly well tolerated. One of the most common reported side effects is bone pain (which is not treatment-limiting), neutrophilic dermatoses, exacerbation of psoriasis and isolated anaphylactic reactions as well as coagulation abnormalities also may appear. Transient renal and biological disturbances are reported [22,23]. Dyspnea, pain in chest and hypoxemia, nausea, diaphoresis, anaphylactic reactions, syncope and flushing are evidenced [24]. Unfortunately, G-CSF is helpless to fight other than leukopenia side effects [25]. Also, it does not penetrate through the alveolar barrier and cannot prevent lung injury, especially during the concomitant radiation therapy [26]. So, it means we may enhance the efficacy of G-CSF therapy in the patients with malignancies during chemotherapy courses and Sorption Detoxification is a promising issue. First positive results for myeloprotective effect of enterosorption were published in 1980-90th [15]. Since that time there are a lot of evidences for successful use of enterosorption in oncology practice [12, 13, 27–29]. Our previous experiments in healthy rats demonstrated advantages of combination of G-CSF and enterosorption to ameliorate side effects of melphalan and cisplatin [16,30]. This study has proved that such combination has no negative impact on tumoricidal activity of alkylating cytostatic agent Melphalan and promotes animals' survival: mortality rate decreased from 57 to 14%.

Conclusions

The side effects of anti-cancer drugs, espe_ cially bone marrow suppression and neutro_ penia, are the main limiting factors for full courses of chemotherapy, which is crucial for treatment efficacy.

Melphalan at single injection at the dose of 5.5 mg/kg caused significant leukopenia and granulocytopenia in the Guerin carcinoma grafted rats. Mortality rate was 57.1%. The filgrastim (recombinant granulocyte colony

stimulating factor) or enteral sorption therapy with carbon oral adsorbent C2 (bulk density γ=0.18 g/cm^3, diameter of granules 0.15-0.25 mm, BET pore surface – 2162 m^2/g) for correction of melphalan-induced myelosuppression increased white blood cell count, but the most prominent results were evidenced only when combination of both factors was used, especially regarding the granulocytes number. Also, only in the group of rats, which received both Filgrastim or r-GCSF, produced by IEPOR, and enterosorption, the restoration of platelets count was observed. The use of both factors of correction did not ameliorate the anti-tumor efficacy of alkylating cytostatic agent.

Thus, the results are perspective for further study of the use of combination of carbon oral adsorbents and hematopoietic cytokines to ameliorate anti-cancer chemotherapy side effects, as well as their implementation into clinical practice.

Conflicts of Interest

Authors declare no conflict of interest.

ЕФЕКТИВНІСТЬ ЗАСТОСУВАННЯ ПРЕПАРАТІВ ГРАНУЛОЦИТАРНОГО КОЛОНІЄСТИМУЛЮЮЧОГО ФАКТОРУ ТА ЕНТЕРОСОРБЦІЇ ПРИ МЕЛФАЛАН-ІНДУКОВАНІЙ МІЄЛОСУПРЕСІЇ У ЩУРІВ З ПЕРЕВИВНОЮ КАРЦИНОМОЮ ГЕРЕНА

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2 – ІНСТИТУТ ЕКСПЕРИМЕНТАЛЬНОЇ ПАТОЛОГІЇ, ОНКОЛОГІЇ І РАДІОБІОЛОГІЇ ІМЕНІ Р.Є. КАВЕЦЬКОГО НАН УКРАЇНИ, КИЇВ, УКРАЇНА

3 – ІНСТИТУТ ЯДЕРНИХ ДОСЛІДЖЕНЬ НАН УКРАЇНИ, КИЇВ, УКРАЇНА

Вступ. *Побічні ефекти протипухлинних лікарських засобів (найчастіше лейкопенія та нейтропенія) слугують основним лімітуючим фактором ефективного лікування. Наші попередні дослідження на здорових тваринах продемонстрували виражену ефективність при мелфалан-індукованій мієлосупресії, однак питання впливу будь яких додаткових чинників, які застосовуються у онкологічній практиці, на ріст та розвиток пухлини надзвичайно важливе.*

Мета роботи: *дослідити дію біосимілярів гранулоцитарного колонієстимулюючого фактора (Г-КСФ) та гранульованого вуглецевого ентеросорбента С2 при мелфалан-індукованій мієлосупресії у щурів з перевивною карциномою Герена.*

Методи. *Для індукування мієлосупресії у щурів з перевивною карциномою Герена мелфалан вводили у дозі 5,5 мг/кг. Для корекції викликаних змін використовували філграстим та аналог виробництва ІЕПОР – рекомбінантний гранулоцитарний колонієстимулюючий фактор (р-ГКСФ). Для проведення ентеросорбції використано гранульований вуглецевий ентеросорбент С2 з питомою вагою γ=0,18 г/см^3, діаметром гранул 0,15–0,25 мм, поверхня пор за BET – 2162 м^2/г). Тварин виводили з експерименту на 17-у добу після перещеплення пухлини (на 8-у добу після введення мелфалану).*

Результати. *Алкілуючий цитостатик викликав глибокі лейкопенію (падіння на 95,7%), нейтропенію (зниження на 73,9%) та тромбоцитопенію (падіння показника на 84,9%) у дослідних тварин. Летальність склала 57%. Застосування препаратів Г-КСФ та ентеросорбента С2 покращувало досліджувані показники, однак найбільш виражене покращення спостерігалося лише при введенні обох чинників разом. Введення обох чинників не зменшувало протипухлинну активність мелфалану як при моновведенні, так і в комбінації.*

Висновки. *Отримані нами результати свідчать про перспективи подальшого вивчення ефективності застосування комбінації ентеросорбції та гемостимулюючих цитокінів для пом'якшення побічних ефектів протипухлинної хіміотерапії та їх впровадження у клінічну практику.*

КЛЮЧОВІ СЛОВА: **мелфалан; карцинома Герена; щури; гранулоцитарний колонієстимулюючий фактор; ентеросорбція.**

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ANTHROPOMETRY OF THE EXTERNAL EAR AMONG ADULT IJAWS IN BAYELSA STATE OF NIGERIA

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Background. *External ear measurement is of utmost importance in reconstructive surgeries.*
Objectives. *The present study is aimed at ascertaining sexual dimorphism in external ear anthropometry and ear lobe attachments among adults Ijaws in Bayelsa, Nigeria.*
Methods. *A total of 112 adults within the age range of 18-50 yrs, who met the inclusion criteria, were involved in the study. Four linear dimensions of the ear, which are ear length (EL), ear width (EW), lobular length (LL) and lobular width (LW), were measured for both genders. The lobular attachment for both ears for males and females were also examined and results recorded. These data were subjected to Student t-test, Chi-square test, and Pearson's correlation using SPSS version 20.0.*
Results. *The mean values for EL, EW, LL, and LW for the left auricle in the males and females were 58.14±0.60, 27.41±0.37, 14.47±0.27, 13.50±0.34 and 57.90±0.16, 27.45±0.65, 15.41±0.31, 13.43±0.38 respectively. However, for the right auricle in the males and females, the values were 58.40±0.45, 28.21±0.68, 14.32±0.31, 13.04±0.32, and 56.66±1.10, 27.51±0.65, 15.58±0.29, 13.28±0.34 respectively. The left and right lobular length were the only parameters that proved statistical significance ($p<0.05$) in females compare to males. Pearson's correlations between right and left sides for each of the parameters were positive and significant. Chi-square analysis revealed no significant relationship ($p>0.05$) between earlobe attachments and gender.*
Conclusions. *Sexual dimorphism was thus established in the Ijaw population as regards lobular length dimensions. It is believed that the results of this study would be very useful for ear morphology and reconstructive surgeries.*
KEY WORDS: **external ear (auricle); Bayelsa Ijaws; anthropometry; sexual dimorphism; lobular attachment.**

Introduction

Anthropometry refers to the branch of anthropology concerned with comparative measurements of the human body for the purpose of understanding of physical variations in humans [1]. These body measurements have been shown to vary according to sex, age and race, thus the study of these physical variations is of utmost importance in plastic surgery, prosthetics, clothing designs, and ergonomics [2-3]. It is also important for development of personal individuality [2].

The human external ear is an important organ contributing to the esthetics of human face. Its size, shape, position, and projection influence the facial appearance of the individual [4]. It consists of external, middle and internal parts. The external ear is made up of a shell-like auricle (pinna), which collects sound, and the external acoustic meatus, which conducts sound from the pinna to the tympanic membrane [5]. The auricle, helical in shape, is made up of an elastic cartilage covered by skin and has several depressions and elevations. The lobule (earlobe) of the auricle is a tag of skin containing fibrofatty tissue and blood vessels and is easily pierced for taking blood samples and inserting earrings [5,6]. It is the last part of the auricle to develop [7]. Earlobe attachments were mainly classified into attached and free (unattached) earlobes [8-12]. Many authors, however, have pointed out that there are many people with intermediate (or tapering) earlobes [13-14]. Individuals having earlobes that hang freely are known to have free (unattached) earlobes, whereas those having earlobes fused with the sides of proximal head are termed as attached (or adherent) earlobes [15-16].

Several anthropometric studies on ear measurements have been carried out on different populations. According to the studies

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carried out in India and Urhobo people of Southern Nigeria, females were found to have a statistically higher lobular length and width compare to males [3,17]. The studies in American, Italian, Indian, Israeli and Turkish populations suggested that boys and men had larger ears than girls and women [8, 19-26]. Similar studies have shown that auricular dimensions in northern Nigeria are higher in males compare to females [27]. The reports by Sharma [3], however, documented that ear dimensions were higher in males than females [3,17]. The studies by Wang, *et al* [18] of a Chinese population, however suggested that lobular dimensions were not significantly different in both genders.

Despite a large number of world publications on the anthropometric measurement of the external ear, the studies correlating the dimensions of the external ear and sexual dimorphism in adult Nigerian population show paucity of information. Thus, the purpose of this study is to determine sexual dimorphism in mean ear dimensions and earlobe attachments among adult Ijaws in Bayelsa State of Nigeria.

Previous studies on the morphometric analysis of the external ear have been carried out previously in several countries including Nigeria, however, the studies correlating the dimensions of the external ear and sexual dimorphism in adult Bayelsa Ijaws show paucity of information and have necessitated the need for this study. Hence the present study aims to ascertain the anthropometry of the external ear among adults Ijaws in Bayelsa state of Nigeria.

The objectives of this study are:

To determine the mean dimensions of ear length (EL), ear width (EW), lobular length (LL) and lobular width (LW) among Bayelsa Ijaws.

To ascertain if there may exist a sexual dimorphism among the parameters mentioned above.

To ascertain if there is any correlation in the measured parameters of the external ear in sexual dimorphism.

To determine the variation in attachment of earlobe among Ijaws.

Methods

The study involved the subjects from various local governments in Bayelsa state: Yenagoa, Sagbama, Southern Ijaw, Nembe, Kolokuma, Ekeremor and Ogbia local government area. The study was conducted on adult Ijaws, whose parents were indigenes of Bayelsa state in Nigeria up to their second generation. The sampling method used for the selection of subjects was a simple random sampling method. A total of 112 subjects were involved: 59 males and 53 females. Only the subjects within the age range of 18-50 years of the Ijaw ethnic group in Bayelsa state without anomalies to their external ears comprised this study. The subjects with ear defects and those with previous history of ear surgery were excluded from the study. Also the subjects, who had not given their consent, were also excluded from the study.

The procedure for ear measurements was explained to the subject in order to get maximum cooperation. With the subject's head in Frankfort horizontal plane, direct measurements were taken using a digital vernier caliper with resolution of 0.01 mm as follows [3]. The anthropometric parameters of the external ear measured were (Fig. 1):

Total length of ear (TLE) – measured as the distance between the superior projection of the helix and inferior projection of the external ear (AB).

Total width of ear (TWE) – measured as the distance between the anterior and superior points of the external ear (CD).

Total lobular width (TLL) – measured from the midpoint of the intertragic notch to the lowest point of the lobule (GB).

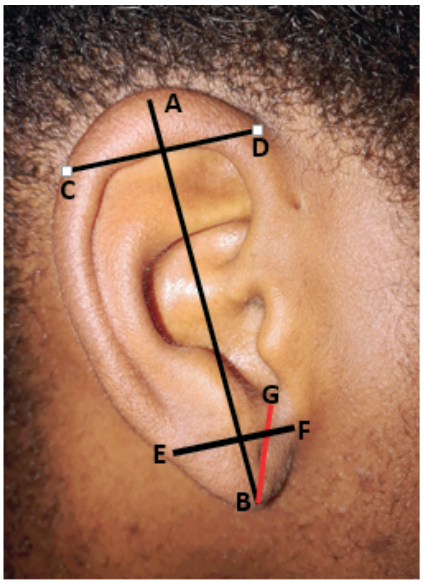

Fig.1. Photograph of the lateral surface of the auricle displaying the measured parameters.
Notes: AB – total length of ear;
CD – total width of ear;
BG – total lobular length;
EF – total lobular width.

Total lobular width (TLW) – measured as the transverse distance of the ear lobule passing through the center of the length of the lobule (EF).

Data analysis was performed using Statistical Package for Social Sciences (SPSS) version 20.0 with results expressed as mean and standard error of mean (Mean ± SE). The Student independent sample t-test with a two-tail distribution was applied to compare the ear measurements between males and females in the study population. P-values, less than 0.05 ($p<0.05$) were considered to be significant. Chi-square test was used to determine the relationship between gender and left lobular attachment of the population. Pearson's correlation coefficient test was conducted to determine the symmetry between the right and left side ear measurements of each gender and for the study population.

Correlation coefficient (*r* value) was interpreted as follows:

a. <0.2 – a slight correlation
b. 0.2–0.4 – a weak correlation
c. 0.4–0.7 – a moderate correlation
d. 0.7–0.9 – a high correlation
e. >0.9 – an almost perfect correlation

Results

The distribution of the subjects involved in the study according to the various Local Government areas of Bayelsa State is presented in Table 1.

Table 1. Detailed analysis of the subjects according to gender and local government areas (LGA) in Bayelsa State.

S/N	LGA	Gender	
		Female	Male
1	Ekeremor	11	6
2	Kolokuma	4	6
3	Nembe	9	2
4	Ogbia	2	4
5	Sagbama	5	11
6	Southern Ijaw	15	17
7	Yenagoa	8	12
	TOTAL	54	58

Seventeen of the subjects hailed from Ekeremor, 10 from Kolokuma, 11 from Nembe, 6 from Ogbia, 17 from Sagbama, 20 from Yenagoa, 27 from Southern Ijaw Local Government Area of Bayelsa State. The result prove that out of the total number of the subjects, who consented and participated in the study,

Table 2. General characteristics of the study population (n=112)

Variables	Mean	SEM	SD	Range	Min	Max	Variance
Age	26.69	0.71	7.56	32.00	18.00	50.00	57.08
LEL	58.02	0.38	4.02	30.34	39.77	70.11	16.12
LEW	27.43	0.36	3.86	36.33	19.97	56.30	14.91
LLL	14.93	0.21	2.18	11.12	9.12	20.24	4.76
LLW	13.47	0.25	2.69	16.85	4.40	21.25	7.24
REL	57.56	0.58	6.16	53.20	16.20	69.40	37.96
REW	27.87	0.47	4.98	39.78	19.98	59.76	24.77
RLL	14.92	0.22	2.34	13.28	9.56	22.84	5.47
RLW	13.15	0.23	2.45	10.68	7.60	18.28	6.02

Notes: REL – right ear length; LEL – left ear length; REW – right ear width; LEW – left ear width; RLL – right lobular length; LLL – left lobular length; RLW – right lobular width; LLW – left lobular width; SEM – standard error of mean; SD – standard deviation.

Table 3. Comparison of the measurements of the study population (n=112).

Variable	n	T	P	Inference
Left ear length-right ear length	112	0.788	0.433	NS
Left ear width-Right ear width	112	-0.813	0.418	NS
Left lobular length-right lobular length	112	0.004	0.997	NS
Left lobular width-right lobular width	112	2.011	0.047	NS

Notes: P – the statistical significance was determined using paired sample T-test; $P<0.05$ – significance between mean values; n – sample size; NS – not significant; S – significant.

54 are females and 58 – males (Table1). The mean values, standard deviation, standard error and variance for each variable of each ear measured in the study population (n=112) are enlisted in Table 2. The results also proved that values for the right ear measurements did not significantly differ (p>0.05) from that on the left (tables 2 & 3) for the study population.

The results of the population data subjected to Chi-square analysis at 95% and 99% confidence interval is presented in Table 4.

The results attained proved that there was a weak correlation (r=0.3) in the left and right ear length of the study population (n=112). The *P* value for the correlations was found to be <0.05 (P=0.001), hence the correlation was statistically significant. Left and right ear width however showed a slight correlation, which was not significant (P=0.073; r=0.2). Significant correlations were found in the study population between the right and left side of lobular length (P=0.000; r=0.7) and that of lobular width (P=0.000; r=0.7).

In Tables 5, 6, and 7, four important indices (ear width, ear length, lobular length and lobular width) were assessed and compared in both and within the sexes. It was evidenced that females had a significantly larger dimensions (p<0.05) of left lobular length and right lobular length compare to that of males (table 5).

The dimensions for left lobular length and right lobular length were 15.41±0.31 and 15.58±0.29 in females, whereas in males – 14.47±0.27 and 14.32±0.31 respectively. Apart from the left and right lobular length that proved higher mean values in females, the mean values of other parameters were not consistently higher (p>0.05) in one gender with respect to a side (Table 5). Within the male population, the right ear measurements did not significantly statistically differ (p>0.05) from that on the left (Table 6).

Table 4. Pearson correlation of right and left side measurements of the study population (n=112).

Variable	n	R	r^2	P
Left ear length-right ear length	112	0.307	0.094	0.001**
Left ear width-Right ear width	112	0.170	0.029	0.073
Left lobular length-right lobular length	112	0.654	0.428	0.000**
Left lobular width-right lobular width	112	0.799	0.638	0.000**

Notes: n – sample size; r – coefficient of correlation; p – statistical significance; superscript * – correlation at 0.05 level (p<0.05); ** – correlation at 0.01 level (p<0.01).

Table 5. Comparison of ear measurements between the male and female study population.

Variable	Sex	n	Mean±SE	T value	P value	Inference
Left ear length	Male	58	58.14±0.60	0.318	0.751	NS
	Female	54	57.90±0.46			
Left ear width	Male	58	27.41±0.37	-0.055	0.956	NS
	Female	54	27.45±0.65			
Left lobular length	Male	58	14.47±0.27	-2.327	0.022	S
	Female	54	15.41±0.31			
Left lobular width	Male	58	13.50±0.34	0.125	0.901	NS
	Female	54	13.43±0.38			
Right ear length	Male	58	58.40±0.45	1.505	0.135	NS
	Female	54	56.66±1.10			
Right ear width	Male	58	28.21±0.68	0.747	0.457	NS
	Female	54	27.51±0.65			
Right lobular length	Male	58	14.32±0.31	-2.954	0.004	S
	Female	54	15.58±0.29			
Right lobular width	Male	58	13.04±0.32	-0.508	0.613	NS
	Female	54	13.28±0.34			

Notes: values are presented as Mean±SE; P – statistical significance was determined using independent sample T-test; P<0.05 – statistical significance between mean values; SE – standard error; n – sample size; NS – not significant; S – significant.

Table 6. Comparison of measurements in males (n=58).

Variable	n	T	P	Inference
Left ear length-right ear length	58	-0.443	0.660	NS
Left ear width-Right ear width	58	-1.180	0.243	NS
Left lobular length-right lobular length	58	0.543	0.589	NS
Left lobular width-right lobular width	58	1.920	0.060	NS

Notes: P – statistical significance was determined using paired sample T-test; P<0.05 – significance between mean values; n – sample size; NS – not significant; S – significant.

Similarly, the right ear indices (right ear length, right ear width, right lobular length, right lobular width) did not statistically significantly differ (p>0.05) from that of the left (left ear length, left ear width, left lobular length, left lobular width) in female population (Table 7).

Tables 8 and 9 present the relationship between the lobular attachments of the ear in males and females subjects of Ijaw ethnicity. The proportion of the male subjects, whose left lobular attachment is either attached, free (unattached) or intermediate, were 22 (19.6%), 17 (15.2%) and 19 (17.0%) respectively (Table 8).

For female subjects, the proportions were 27 (24.1%), 8 (7.1%) and 19 (17.0%) respectively. Results proved no significant relationship between the left lobular attachment and gender (X^2=3.612; P=0.164). The proportion of the male subjects, whose right lobular attachment is either attached, free or intermediate, were 21 (18.8%), 11(9.8%), and 26 (23.2%) respectively (Table 9). On the other hand, the proportion of the female subjects, whose right lobular attachment was either attached, free or intermediate, were 14 (12.5%), 6 (5.4%), and 34 (30.4%) respectively. There was no significant

Table 7. Comparison of the left and right ear measurements in females (n=54).

Variable	N	T	P	Inference
Left ear length-right ear length	54	1.189	0.240	NS
Left ear width-Right ear width	54	-0.660	0.948	NS
Left lobular length-right lobular length	54	-0.813	0.420	NS
Left lobular width-right lobular width	54	0.800	0.427	NS

Notes: P – statistical significance was determined using paired sample T-test; P<0.05 – statistical significance between mean values; n – sample size; NS – not significant; S – significant.

Table 8. Relationship between gender (sex) and left lobular attachment of the population (n=112).

Sex	Left lobular attachment			Total	Chi-square analysis		
	Attached	Free	Intermediate		df	X^2	p-value
Male	22 (19.6%)	17 (15.2%)	19 (17.0%)	58 (51.8%)	2	3.612	0.164
Female	27 (24.1%)	8 (7.1%)	19 (17.0%)	54 (48.2%)			
Total	49 (43.8%)	25 (22.3%)	38 (33.9%)	112 (100.0%)			

Notes: the values are presented as frequencies and percentages; P<0.05 – the association (or relationship) between variables.

Table 9. Relationship between gender (sex) and right lobular attachment of the population (n=112).

Sex	Left lobular attachment			Total	Chi-square analysis		
	Attached	Free	Intermediate		df	X^2	p-value
Male	21 (18.8%)	11(9.8%)	26 (23.2%)	58 (51.8%)	2	3.799	0.150
Female	14 (12.5%)	6 (5.4%)	34 (30.4%)	54 (48.2%)			
Total	35 (31.3%)	17(15.2%)	60 (53.6%)	112 (100.0%)			

Notes: the values are presented as frequencies and percentages. P<0.05 means there is an association (or relationship) between variables.

relationship between gender and right lobular attachments (X^2=3.799; P= 0.150).

Discussion

The current study presents anthropometric data regarding the external ear measurements for individuals of Bayelsa State, Nigeria. Gender (sexual) dimorphism was presented in the study as regards the measurement of lobular length. The results revealed that lobular length (left and right) were significantly higher ($p<0.05$) in female subjects compared to that in males. These findings are supported by other studies carried out involving India and Urhobo people of Southern Nigeria, where females were also found to have a statistically higher lobular length than males [3,17]. Our findings however contradict with reports in northern Nigeria which showed that lobular dimensions were higher in males compare to females [27]. When the mean value for lobular length, measured on both sides with respect to gender, of our study was compared with other anthropometric studies in Nigeria, it was evidenced that Urhobo people of Southern Nigeria had similar mean values, while the residents of Maiduguri in Northern Nigeria had lower values. This implies that within Nigeria, people of different ethnicity and geopolitical zones have different dimensions of Lobular length. The findings by Wang et al. [18], in a Chinese population, however suggested that lobular dimensions were not significantly different in the two genders. The disparity in mean ear dimensions, across subjects in Nigeria as well as other countries of the world, could be attributed to differences in social and ethnic background.

The data of the present study was analyzed using Pearson correlation and it was observed that all parameters on both sides of the ear proved to have a positive linear relationship, which was highly significant ($P<0.05$). This was similar to the findings of Ekanem et al. [4] and Eboh [17].

Analyzing the data with regards to sex of the subjects, it was observed that ear length and width did not vary significantly ($p>0.05$) between males and females. Our findings however contradict with the reports in northern Nigeria, which showed that auricular dimensions were higher in males compare to females [4]. Similarly, our findings also contradicts with reports of Azaria et al. [8], Sharma et al. [9], Brucker et al. [19], Bozkir et al. [22] Purkait and Singh [27], Ito et al. [28], and Meijerman et al. [29], who suggested that males have longer ears compare to females. The disparity in mean ear dimensions across the subjects in different countries of the world could be associated with difference in social and ethnic background.

Various authors have studied the attachment of ear lobules in different populations and its significance in population genetics [11, 15]. The results of the present study, from data analyzed using Chi-square test, revealed no association ($p>0.05$) between gender and lobular attachment for both ears. The frequency of attached, free and intermediate ear lobule in Ijaw population of Bayelsa State, Nigeria, were 43.8%, 22.3% and 33.9% respectively for the left ear and 31.3%, 15.2% and 53.6% respectively for the right ear. In other words, attached and intermediate ear lobules were found to be the predominant ($p>0.05$) type of ear lobule in the Ijaw population of Bayelsa State, Nigeria. Our study corroborates with findings by Munir et al. [15], Shah et al. [30], Kim et al. [31], who revealed a higher frequency of attached and intermediate ear lobules in Japanese/Chinese and Indian population respectively. In a similar research carried out by Munier et al. [15], it was proved that no association ($p>0.05$) existed between ear lobular attachment and gender. The present study however differs from the findings by Nwaopara et al. [32] and Singh and Sengupta [33], who have reported a higher frequency for free (unattached) ear lobule (63.4%) amongst the population of Ekpoma, Nigeria and Assamese, India respectively.

Conclusions

The present anthropometric study on ear measurements revealed a significantly higher lobular length in females compare to males, thus sexual dimorphism was established as regards the measurement of lobular length in Ijaws population. Other parameters such as ear length, ear width, lobular width, and lobular attachment did not exhibit significant gender differences in the study population. There was a high correlation in the dimensions of ear length, lobular length, and lobular width of the pinna between the right and left sides in the study population. It is believed that the results of this study would be very useful in ear morphology and reconstructive surgeries.

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Conflict of interest
The authors declare no conflict of interest.

АНТРОПОМЕТРИЧНІ ДОСЛІДЖЕННЯ ЗОВНІШНЬОГО ВУХА СЕРЕД ДОРОСЛОГО НАСЕЛЕННЯ ІДЖО ШТАТУ БАЙЕЛСА У НІГЕРІЇ

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Вступ. *Вимірювання зовнішнього вуха є надзвичайно важливим у морфології вуха та реконструктивній хірургії.*

Мета роботи – *дослідити статевий диморфізм у антропометрії зовнішнього вуха (довжина вуха, ширина вуха, висота вушної сережки (мочки), ширина мочки) та прикріплення мочки серед дорослого населення етнічної групи іджо у штаті Байелса, Нігерія.*

Методи. *Обстежено 112 дорослих віком від 18 до 50 років, які відповідали критеріям дослідження. Чотири лінійних виміри зовнішнього вуха, а саме довжина вуха (ДВ), ширина вуха (ШВ), довжина мочки (ДМ) та ширина мочки (ШМ) були проведені у досліджуваних осіб обох статей. Прикріплення мочки у чоловіків та жінок також фіксувалося. Отримані дані обробляли з використанням t-критерію Стьюдента, критерію Хі-квадрата кореляції Пірсона за допомогою програмного забезпечення SPSS версії 20.0.*

Результати. *Середні величини для досліджуваних показників ДВ, ШВ, ДМ,ШМ склали у чоловіків та жінок 58,14±0,60, 27,41±0,37, 14,47±0,27, 13,50±0,34 та 57,90±0,16, 27,45±0,65, 15,41±0,31, 13,43±0,38 відповідно. Однак для правого вуха у чоловіків і жінок ці показники були 58,40±0,45, 28,21±0,68, 14,32±0,31, 13,04±0,32 та 56,66±1,10, 27,51±0,65, 15,58±0,29, 13,28±0,34 відповідно. Статистично достовірні відмінності у розмірах були лише у показника довжини лівої і правої мочки у жінок відносно до чоловіків ($p<0.05$). Коефіцієнт кореляції Пірсона між параметрами правого та лівого вуха був достовірним та позитивним. За критерієм Хі-квадрата не було знайдено достовірних взаємозв'язків ($p>0.05$) у прикріпленні мочки вуха у різних статей.*

Висновки. *Проведене нами дослідження дозволило встановити статеві відмінності у довжині мочки вуха у популяції Іджо. Ці дані будуть корисними при вивченні морфології вуха та при проведенні реконструктивних втручань на вусі.*

КЛЮЧОВІ СЛОВА: **зовнішнє вухо(*auricla*); Іджо штату Байелс; антропометрія; статевий диморфізм; прикріплення мочки вуха.**

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ANTIOXIDANT ENZYMES ACTIVITY IN EXPERIMENTAL ISCHEMIA-REPERFUSION INJURY

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Background. *Blood loss during civil and military limb trauma is the most common cause of preventable death. Complications due to the use of a hemostatic tourniquet are widely investigated nowadays. Therefore, the standards of the past have to be improved.*

Objective. *The aim of the research is to study the reaction of the enzyme chain of the liver antioxidant system in the presence of modifications of ischemia-reperfusion injury (IRI).*

Methods. *210 white male-rats, aged 5-5.5 months, were used in the research. The dynamics of antioxidant enzymes activity catalase (Cat) and superoxide dismutase (SOD) in liver tissue in cases of modifications of ischemia-reperfusion injury (IRI) were studied. The period of investigation was in 24 hours, 3, 7, 14 days after the injury.*

Results. *In cases of simulated IRI the catalase level mainly decreased at each period of the experiment. The peak of SOD activity was evidenced on the 1st, 3rd or 7th days after the experimental IRI according to the degree of trauma severity. Thus, IRI combined with severe blood loss and mechanical trauma caused the severest affection of the antioxidant system. Even a single application of hemostatic tourniquet caused similar wavelike reactions at different times.*

Conclusions. *The development of IRI is accompanied by a significant depression of the liver antioxidant system. The most significant changes were evidenced in cases of IRI combined with blood loss and mechanical trauma, but even a single application of a tourniquet caused active response of the antioxidant enzymes.*

KEY WORDS: **ischemia-reperfusion injury; trauma; blood loss; hemostatic tourniquet; catalase; superoxide dismutase.**

Introduction

The damage to both skeletal muscles and internal organs caused by local ischemia-reperfusion injury (IRI) is mostly present as a result of intraoperative use of tourniquet for temporary stop of blood circulation in internal organs or for stop of blood loss from the limb [1-10]. Primary damage leads to mechanical pressure, as for secondary mechanisms – reactive oxygen intermediates and lipid peroxidation are responsible for it [11]. Nowadays, in state of affairs of Ukrainian hostilities, gunshot wounds are widespread, and use of hemostatic tourniquet is one of efficient methods. But complications accompanying it are more serious than it was though previously.

In the case of the hemostatic tourniquet use it is important to know about period of depression of antioxidant system in vitally important internal organs, for preventing exhaustion in further period consequences.

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Oxidative stress is crucial in development of local and systemic damage and progress of ischemia-reperfusion injury in cases of oxygen insufficiency of the tissues [12]. Overproduction of reactive oxygen species (ROS) is combined with the violation of oxidative-reduction systems activity, damage of DNA, membrane receptors, dysfunction of ion channels and changes in composition of membrane phospholipids, as well as activation of caspase mechanism of apoptosis [13, 14, 15]. All this is leading to activation of endogenous antioxidant defense [13, 16-22].

Cellular systems of antioxidant protection are classified as enzymatic and non-enzymatic. The first one includes superoxide dismutase (SOD), glutathione peroxidase (GP), and catalase (Cat), which provide the first line of defense against the action of ROS in that way, when the product of the first reaction becomes a substrate for the next one [23].

Overexpression of SOD1 prevents neuronal death in the area of hippocampus [24]. However, the extremely short period of half-life of SOD1 in circulating blood makes it difficult to use enzyme therapy for brain damage. In

some studies GP provides even greater defense against oxidative stress than SOD – its expression, as well as SOD provides cytoprotective effect [16, 18]. Cat is a very important element in maintaining the intracellular concentration of reduced glutathione and is crucial in neutralization of free radicals [24, 25].

Various methods are used nowadays in therapeutic treatment: from lowering the temperature of the limb to the use of various drugs. Thus, the protective effect of curcumin on the myocardium, kidneys, nervous tissue and lung has been proved in cases of this pathology [26-29]; montelucast reduces the level of local and systemic manifestations [29], legalon, thiotriazolin, emoxipine, silymarin have antioxidant effect [3-9, 25-30]. Studying of changes in the activity of antioxidant enzymes is needed for prediction of the effect of a specific antioxidant corrector. This is essential to avoid exhaustion of the impaired body systems.

Objective. The aim of the research was to study the reaction of enzyme chain of the liver antioxidant system in the presence of modifications of IRI.

Methods

The experiments were performed on 210 non-linear white male rats, 250-270 g in weight and 5-5.5 months of age. They were divided into 5 groups, each of them contained 10 animals: the control group involved rats, which were administered thiopental-sodium anesthesia (40 mg/kg of body weight intramuscularly) only, the **1st experimental group** (tourniquet was applied to the upper thigh 1/3 for 2 hours, reperfusion for 1 hour; the **2nd experimental group** (blood loos in amount of 40% of circulating blood volume was simulated; the **3rd experimental group** (a tourniquet on a thigh was combined with 40% blood loss from femoral vein of another lower limb), the **4th experimental group** (a tourniquet on a thigh was combined with femoral bone fracture of another lower limb), the **5th experimental group** (a tourniquet on a thigh was combined with 40% blood loss and femoral bone fracture of another lower limb).

The experiments were performed in the vivarium of I. Horbachevsky TNMU in the morning. Special room had stable temperature (18-22 °C), relative humidity (40-60%) and illumination 250 lux. Animals were sacrificed on the 1st, 3rd, 7th and 14th days after the trauma by thiopental-sodium anesthesia (40 mg/kg of body weight intraperitoneally by total bloodletting from the heart. An activity of catalase (mckat/kg) and superoxide dismutase (U/mg) of 10% liver homogenate samples were determined by means of Koroliuk MA, et al. (1988) and Chevari S, et al. (1985) methods respectively.

All experimental stages of the research were performed following the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986), resolution of the First National Congress on Bioethics (Kyiv, 2001) and the Order of the Ministry of Health of Ukraine No. 690, dated September 23, 2009.

A statistical analysis of the attained data was performed by Excel (Microsoft, USA). Statistically significant differences between the independent indices were estimated by the Student t-test at normal distribution and by nonparametric methods in other cases. The results were presented as (M±m), where M means value, m – standard error. Correlation analysis was performed for the attained data. Linear correlation coefficient (r) and its significance (b) were evaluated as well. The link was considered to be lost if the r index was 0, the link evidenced of a week correlation when the range was 0-0.03. In the case of the range 0.3-0.7 – a medium link was established, the range of 0.7-1.0 proved a strong interaction correlation. The correlation coefficient was significant at $p<0.05$.

Results

The total content of the investigated enzymes of the liver antioxidant system is presented in Table 1.

On the 1st day, compare to the control, an increase of SOD in 3.1 times ($p1<0.01$), by 80% ($p2<0.01$), by 13% ($p3<0.002$), in 70% ($p4<0.003$), in 4.6 times ($p5<0.006$) was evidenced. As for Cat, its level decreased the most. Thus, on the 1st day its decrease in 24.1 times ($p1<0.01$), in 13.5 times ($p2<0.01$), in 17.8 times ($p5<0.004$) took place as well as an increase by 16% ($p3<0.004$) and 38.6% ($p4<0.002$).

On the 3rd day, compare to the control, in all experimental groups there was an total increase of SOD activity in 3 times ($p1<0.003$), in 5.4 times ($p2<0.007$), in 3.6 times ($p3<0.03$), in 2.48 times ($p4<0.01$), by 86% ($p5<0.007$). As for Cat, its dynamic was variable. A decrease in its level in 24 times ($p1<0.003$), in 11.9 times ($p2<0.005$) was evidenced, but in cases of other traumas – an increase of its level in 1.8 times

(p3<0.3), by 77.6% (p4<0.05), in 1.8 times (p5<0.08).

On the 7th day, compare to the control, there was an evident increase if SOD activity in all experimental groups: in 13 times (p1<0.02), in 3.1 times (p2<0.006), in 12.8 times (p3<0.02), in 4.2 times (p4<0.009), in 5.3 times (p5<0.003). In all groups, except for the 3rd one, a decrease of catalase activity was evidenced: in 3.5 times (p1<0.02), in 8.3 times (p2<0.005), in 15.2 times (p4<0.005), in 21.8% (p5<0.02). So, in the 3rd group an increase in 12.8 times (p3<0.02) took place.

The similar dynamic of SOD, compare to the control, was evidenced on the 14th day. An increase in its level in 2.2 times (p1<0.01), in 2.4 times (p2<0.004), in 5.8 times (p<0.006), in 2.36 times (p4<0.007), in 5.4 times (p5<0.01) took place.

As for Cat mostly depression was evidenced: a decrease of activity, compare to the control, in 10.3 times (p2<0.005), in 13.2 times (p3<0.004), in 1.67 times (p4<0.05), in 21.4% (p5<0.04), and a very slight increase of it in cases of isolated tourniquet – by 17% (p1<0.05).

In cases of activation of lipid peroxidation and oxidative stress, according to the literature, different levels of activity of the antioxidant system in all organs of the affected organism take place. The attained results proved that in 1 day in liver homogenate, the SOD activity increased in all experimental groups, but the most– in cases of a tourniquet alone and IRI combined with blood loss and mechanical trauma, when its level, compare to the control, increased in 3.1 and 4.6 times respectively.

In all groups, a wavelike reaction of enzyme activity was revealed. Thus, in cases of isolated IRI on the 1st and 3rd days it increased in 3-3.1 times; it suddenly increased (including the steady rates, compared with the previous periods) in 13 times, compare to the control, on the 7th day – in 13 times; it did not reached the norm on the 14th day. Blood loss only, associated with primarily hypoxia of the ischemic genesis, triggered activation with peak of its activity on the 3rd day; it was still high. The combination of classic IRI with blood loss contained elements of both abovementioned manifestations: a mild increase in the activity (apparently due to the fight against hypoxia) in the presence of blood loss only as well as in cases of IRI only; the peak of increased SOD activity on the 7th day reached 12.8-fold rate compare to the control.

The activity of SOD in cases of IRI combined with mechanical trauma increased and decreased very smoothly with its peak on the 7th day, then it did not reached a normal level on the 14th day, being higher than the initial level in 2.36 times, and lower than the previous period index by 44%.

In cases of IRI combined with blood loss and mechanical trauma, two peaks of the SOD activity increase were evidenced: on the 1st day (in 4.6 times, compare to the control) and in 5.3 times on the 7th day, compare to the control. On the 3rd day, which was obviously life-threatening for this type of injury, the index

Table 1. Changes in superoxide dismutase (U/mg) and catalase activity (mckat/kg) in 10% liver homogenates of the studied rats in cases of modifications of ischemic reperfusion injury and isolated blood loss, (M±m).

Group		Indices	1	2	3	4	5
Post traumatic period	Control		Tourniquet T	Blood loss BL	T+BL	T+F	T+BL+F
In 24 hours after trauma	SOD 0.15±0.09	SOD	0.47±0.06*	0.27±0.04*	0.17±0.01	0.26±0.02*	0.70±0.02*
		Cat	0.18±0.08*	0.33±0.07*	5.17±0.02*	6.15±0.01*	0.25±0.02*
In 3 days after trauma		SOD	0.45±0.02	0.82±0.03*,**	0.54±0.16*,**	0.37±0.01*,**	0.28±0.03*,**
		Cat	0.18±0.01*	0.37±0.02*	8.01±1.3*,**	7.89±0.3*,**	8.09±0.36*,**
In 7 days after trauma	Cat 4.44±0.36	SOD	1.94±0.05*,**	0.47±0.03*,**	1.93±0.06*,**	0.29±0.02*,**	0.80±0.02*,**
		Cat	1.28±0.16*,**	0.54±0.02*,**	6.63±0.27*,**	0.63±0.04*,**	3.47±0.05*,**
In 14 days after trauma		SOD	0.32±0.06*,**	0.36±0.03*,**	0.88±0.02*,**	0.35±0.03*,**	0.83±0.06*
		Cat	5.20±0.36*,**	0.43±0.03*	0.34±0.02*,**	2.65±0.20*,**	3.49±0.20*

Notes: * – statistical significance compare to the control, ** – statistical significance compare to the previous period of the study.

slightly exceeded the initial control level, but was statistically significantly lower than on the 1st day (in 2.5 times). On the 14th day it was still increased and the same as on the 7th day.

In cases of IRI only, the dynamics of Cat activity was depressed with a peak of decrease on the 3rd day (in 24 times). In the presence of blood loss only in all study periods, the activity of catalase was statistically significantly lower compare to the control.

The peculiar features of the IRI course combined with blood loss was an increase in the activity of this enzyme on the 3rd day in 1.8 times compare to the control; on the 14th day the index decreased in 13.2 times compare to the control, and in 23.6 times compare to that on the 3rd day.

A somewhat similar dynamics of catalase activity was evidenced in cases of IRI combined with mechanical trauma, when after a gradual increase till the 3rd day, it suddenly decreased in 15.2 times compare to the control on the 7th day. Although, the index was still decreased on the 14th day being lower than the initial level in 1.67 times.

The peculiar features of the Cat dynamics in cases of IRI combined with blood loss and mechanical trauma, was a sudden decrease of its activity on the 1st day compare to the control; then the Cat activity suddenly increased on the 3rd day in 1.8 times compare to the control and was of the initial level up to the end of the experimental period, which obviously proved the reduction of enzymatic component on the 14th day.

Discussion

The correlative analysis proved that, in development of ischemia-reperfusion syndrome caused by application of hemostatic tourniquet, liver failure is significant. This is the focus of the further research on morphological changes of liver tissue. Hepatic active response is a predictable reaction, which belongs to the multiply organ failure syndrome. Association of IRI as the cause of such pathological effect in the organism was proved by a couple of researchers [32-36].

Probably, the depression of SOD is caused by overproduction of malonic dialdehyde and other derivates of peroxidation. Detection of reactive oxygen intermediates accumulation, enzymes of cytolysis and oxidative modification of proteins might be studied in our future experiments, as these indexes are typical in such course of pathological external affection [37, 38]. Moreover, the data on changes in the activity of serum and organ catalase in cases of trauma and blood loss vary. Both decrease and increase of Cat level were presented in different experimental studies [13, 21, 22]. And in our research such fluctuations of this enzyme activity were proved.

In our case active response of the liver antioxidant system was evidenced by an increased activity of antioxidant enzymes. Our studies coincide with those of other researches. E. Orlova *et al*. proved that SOD activity was the highest in liver and they advised correction of its insufficiency with Vin-Vita [23]. Affection of liver tissue caused by application of tourniquet led to possible development of multiple organ failure due to the ischemic-reperfusion limb syndrome [37].

Besides, we consider that IRI combined with mechanical trauma may cause development of abrupt affecting of the liver antioxidant system that was proved by higher level of SOD activity than in other groups and which was still increased till the end of experiment. Such dynamics of SOD and Cat activity is a result of lipid peroxidation, i.e. increase in malonic dialdehyde level [2, 11]. It has been established that reperfusion syndrome affects the development of systemic changes in cases of combined trauma complicated by bleeding that is manifested by a significant activation of lipid peroxidation.

Conclusions

Even a single use of a hemostatic tourniquet leads to significant changes in the activity of enzymatic level of antioxidant defense. In present hostilities, blood loss is often combined with skeletal injuries, so it is advisable to limit the time of use of a hemostatic tourniquet or to take measures that counteract the development of lipid peroxidation caused by an injury in the need of bleeding stopping with a tourniquet.

Conflict of interest

The authors declare no conflict of interest.

ЗМІНА АКТИВНОСТІ ФЕРМЕНТІВ АНТИОКСИДНОГО ЗАХИСТУ ПРИ ЕКСПЕРИМЕНТАЛЬНОМУ ІШЕМІЧНО-РЕПЕРФУЗІЙНОМУ СИНДРОМІ

Н.В. Волотовська, Т.В. Кащак
ТЕРНОПІЛЬСЬКИЙ НАЦІОНАЛЬНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ ІМЕНІ І.Я. ГОРБАЧЕВСЬКОГО, ТЕРНОПІЛЬ, УКРАЇНА

Вступ. *Крововтрата при цивільній та бойовій травмі кінцівок є найпоширенішою причиною т.з. «превентивних смертей». Оскільки стандарти минулого щодо надання невідкладної медичної допомоги для зупинки кровотеч потребують поліпшення, сьогодні широко досліджуються ускладнення при накладанні гемостатичного джгута.*

Мета дослідження. *Вивчити особливості реакції ферментативної ланки антиоксидантної системи печінки на тлі модифікацій ішемічно-реперфузійного синдрому (ІРС).*

Методи дослідження. *У експерименті використано 210 білих щурів-самців віком 5-5,5 місяців. Досліджено динаміку активності антиоксидантних ферментів каталази (Кат) та супероксиддисмутази (СОД) в тканині печінки при розвитку модифікацій ІРС. Забір зразків тканини здійснювали через 24 години, на 3, 7 та 14 доби після травми.*

Результати. *У наших моделях ІРС рівень Кат в основному зменшувався в кожному періоді експерименту. Пік активності СОД спостерігався на 1, 3 або 7 добу після експериментального ІРС – згідно модифікацій ступенів тяжкості травми. Таким чином, застосування кровоспинного джгута в поєднанні з втратою крові та механічною травмою, викликали найважчі ураження антиоксидної системи. При цьому, навіть одноразове застосування джгута викликало подібні хвилеподібні реакції.*

Висновки. *Розвиток ІРС супроводжується сильним пригніченням антиоксидної системи печінки. Найбільш значні зміни спостерігалися на тлі застосування кровоспинного джгута, поєднаного з втратою крові та механічною травмою, але навіть ізольоване застосування джгута викликало суттєву зміну активності антиоксидних ферментів.*

КЛЮЧОВІ СЛОВА: **ішемічно-реперфузійний синдром; травма; втрата крові; гемостатичний джгут; каталаза; супероксиддисмутаза.**

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