International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 5–8

DOI 10.11603/ijmmr.2413-6077.2015.2.6371

EFFECTIVENESS OF CANEPHRON® N IN THE COMPLEX MANAGEMENT OF SUBCLINICAL GOUTY NEPHROPATHY

S. I. Smiyan, M. V. Franchuk, R. R. Komorovsky
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

Background. *The risk of chronic kidney failure increases by 3–10 times with the steady increasing of uric acid level in the blood. It is known that the protein fractions is closely correlated with the level of uric acid.*

Objective. *Microalbuminuria and microglobulinuria are predictors of kidney damage. The study involved 50 patients with gout who had never received preventive treatment of gouty nephropathy. We choosed Canephron N (Bionorica, Neumarkt, Germany) as a combined phytodrug with nephroprotective effect. All studied patients were men with obesity.*

Results. *According to standard examination kidney damage haven't been found, but laboratory tests on microproteinuria showed that the vast majority of patients have signs of subclinical gouty nephropathy.*

Conclusions. *Canephron N in complex gout treatment helps to decrease uric acid level in the blood and increase its excretion.*

KEY WORDS: **gout, chronic kidney disease, hyperuricemia, Canephron N**

Introduction

The term "gouty nephropathy" (GN) comprises all renal pathology that may occur in patients with gout, including urate nephrolithiasis, tophi in the renal parenchyma, glomerulosclerosis, arteriosclerosis with subsequent nephrosclerosis, interstitial nephritis and chronic renal failure. Moreover, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) for symptomatic treatment of patients with gout is associated with nephrotoxicity and may result in acute tubular necrosis, acute interstitial nephritis, proteinuria, hypertension, hyperkalemia [6, 13]. The prevalence of kidney damage in patients with gout ranges from 30 to 70%. Hence it is essential that patients with chronic gout receive therapy for prevention of GN without any (or with minimal) side effects and contraindications. For this purpose we have chosen a herbal based medicine – Canephron® N (Bionorica, Neumarkt, Germany) which is an approved medicinal product containing a fixed combination of centaury herb (Centaurium sp.), lovage root (Levisticum officinale Koch), and rosemary leaves (Rosmarinus officinalis L.) [19]. It has been available on the European market for more than 40 years. The drug has diuretic [12, 33], spasmolytic [1, 32], anti-inflammatory [11, 23, 29], antimicrobial [7, 8, 17], nephroprotective [18] and hypouricemic [25] effects. Some clinical studies show a therapeutic benefit in patients with urinary tract infections [9, 21, 26] and diabetic nephropathy [19].

Corresponding author: ***Svitlana Smiyan, Department of Internal Medicine No 2, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001***
Tel. +380352273377
E-mail: smyjan@tdmu.edu.ua

Materials and Methods

We examined 50 patients with gout (all men), who were hospitalized in the Department of Rheumatology of Ternopil University Hospital. The patients were not previously diagnosedor tested for GN, nor were previously tested for GN. All the patients underwent main laboratory and instrumental methods of investigation and some additional tests for microalbumin and microglobulin levels in morning urine performed by means of ELISA method. Microproteinuria means any manifestation of microalbuminuria (MA), microglobulinuria (MG) or their combinations. The patients were divided into 2 groups: the patients of group I (n=25), the study group, received standard urate lowering therapy (Allopurinol), NSAIDs for pain control and Canephron® N; the patients of group II (n=25), the control group, received only Allopurinol and NSAIDs. Body mass index (BMI), plasma and urine uric acid (UA) levels, blood creatinine, urea and glomerular filtration rate (GFR) were tested. Kidney ultrasound and joint x-ray were also performed. Canephron® N was prescribed, 2 tablets 3 times per day for 6 weeks. After 6 weeks patients' microalbumin, microglobulin levels in urine and UA levels in plasma and urine were re-examined.

Continuous variables are expressed as mean±standard error of the mean. Differences between the variables were determined by unpaired or paired t-test, as appropriate. A value of p<0,05 was considered to be statistically significant.

Results and Discussion

Baseline indicators of the examinations are presented in Table 1. Due to the BMI, the majority of patients in both groups were diagnosed with obesity. Blood creatinine level, urea and GFR were uninformative, because the findings were normal in both groups. Also nephrolithiasis was found in both groups. Laboratory and instrumental investigations showed that a significant proportion of the patients had subclinical GN (fig. 1).

In the study group 48% patients had MA and 60% of them had MG. In the control group 44% patients had MA and 52% of them had MG. The patients from the study group received a combined treatment (Allopurinol, NSAIDs + Canephron N) and had better test results than the group which received standard treatment (Allopurinol, NSAIDs) (Table 2).

The pathogenesis of GN is associated with hyperproduction of UA and imbalance between the processes of its tubular secretion and reabsorption. But currently there is no enough evidence that hyperuricemia (HU) is a marker of renal dysfunction or a risk factor for kidney disorders. The controversial results of the impact of HU on development of chronic kidney disease (CKD) are, partly, due to difficulties in diagnosis of GN at the early stage because of a long subclinical period [5, 10, 24, 27].

Hyperproduction of UA and its excretion decrease leads to HU. The risk of chronic kidney

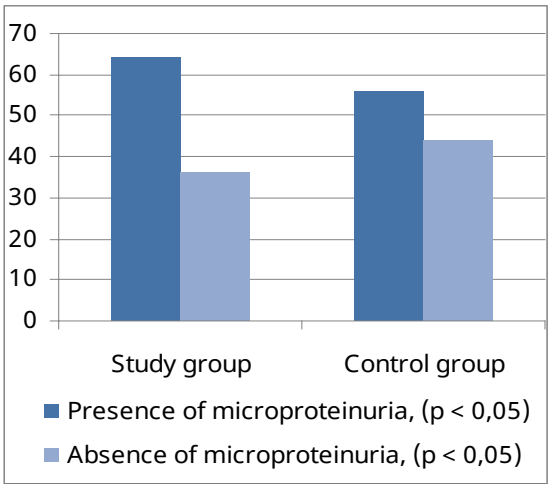

Fig. 1. Microproteinuria in patients suffering from gout.

Table 1. Baseline indicators

Markers	Study group (n=25)			Control group (n=25)			p-value
Age, (yrs)	54,98±1,22			50,63±1,19			<0,05
Disease duration, (yrs)	8,96±0,57			7,42±1,43			>0,05
BMI	28,64±0,61			30,62±1,37			>0,05
Creatinine, (mmol/L)	72,12±0,39			78,31±1,21			>0,05
Urea, (mmol/L)	5,05±0,65			4,96±0,27			>0,05
GFR, ml/min	130,88±1,22			129,64±0,31			>0,05
Uric acid in plasma, (mcmol/L)	0,556±0,08			0,552±0,12			>0,05
X-ray stage, (%)	I	II	III	I	II	III	–
	11,4	79,5	9,1	11,8	76,5	11,7	
Nephrolithiasis, (%)	28			36			–

p – significant differences between the baseline indicators of the study and control groups.

Table 2. Dynamics of changes at the beginning of the study and after 6 weeks of combined treatment with Canephron N and standard therapy (the study group), and standard therapy only (the control group)

Markers	Study group (n = 25)		Control group (n = 25)		p-value
	Baseline	After treatment	Baseline	After treatment	
Microalbuminuria, (mg/L)	131,81±3,45	62,38±2,99*	114,72±3,91	122,19±2,11	<0,05
Microglobulinuria, (mg/L)	20,08±2,06	13,37±3,32*	22,36±3,12	21,19±2,94	<0,05
Uric acid in plasma, (mcmol/L)	0,556±0,08	0,457±0,06*	0,562±0,12	0,498±0,23**	<0,05
Uric acid in urine, (mmol/L/day)	5,21±1,18	6,72±1,34*	5,02±1,09	5,08±1,02	<0,05

p – significant differences between the post-treatment markers of the study and control groups;
* – after treatment in comparison with baseline markers of the study group (p<0,05);
** – after treatment in comparison with baseline markers of the control group (p<0,05).

failure (CKF) rises in 3–10 times caused by increase in UA in blood. Every 4th patient has CRF as a gout complication [3, 4, 5, 22]. Several studies have shown relations of HU to the signs of kidneys damage [30, 31]. Also UA can affect renal hemodynamics due to vasoconstriction in cortical layer and increase the expression of renin. An additional mechanism in kidneys damage is the UA impact on the formation of the endothelial dysfunction by increasing monocyte chemoattractant protein-1 (MCP-1) in vascular smooth muscle fibres and cells of the proximal renal tubules. MCP-1 is a main pathogenetic chemokine of CKD and atherosclerosis [16].

It is established that the protein fraction is closely correlated with the level of UA in blood; that is why HU causes endothelial dysfunction and MA [2]. If albumins and other highmolecular weight proteins are found in urine, glomerular injury is present. Microglobulins (β_2-, α_1- and retinol-binding protein), MG, are tubular disorders characterized by low-molecular weight proteins in urine. MA and MG are predictors of kidneys damage [14, 15, 19, 20, 28]. Reference limits of MA and MG are presented in table 3.

Table 3. Reference limits of microalbuminuria and microglobulinuria

Type	Indicators (mg/L)
Normoalbuminuria	<20
Microalbuminuria	20–200
Macroalbuminuria	>200
Normoglobulinuria	<12
Micro- and macroglobulinuria	>12

Conclusions

Microalbuminuria and microglobulinuria are the main early symptoms of renal damage in patients with gout. The prevalence of kidney damage is significantly ($p<0,05$) higher than the incidence of gouty nephropathy in clinical practice. Blood creatinine level, urea and GFR are uninformative at the stage of GN formation, which is asymptomatic. GN was diagnosed in 64% patients of the study group and in 56% ones of the control group according to the levels of MA and MG. Uncontrolled hyperuricemia, which does not reach the target level, is a major risk factor for development of GN. The group of the patients who received Canephron N as a standard gout treatment had better results after re-examination. This herbal based medicine has uricosuric effect because it decreases UA level in plasma. Microproteinuria decreased in 2 times after the combined treatment with Canephron N. Also we did not detect any side effects during 6 weeks of the study. So, Canephron N is recommended for treatment of subclinical gouty nephropathy.

References

1. Abdul-Ghani AS, El-Lati SG, Sacaan A, et al. Anticonvulsant effects of some Arab medicinal plants. Int J Crude Drug Res 1987; 25: 39–43.

2. Bratus V, Talaia T, Shumakov V. Obesity, insuline resistance, metabolic syndrome: basic and clinical aspects. The fourth wave, Kyiv, 413.

3. Brenner B. The kidney. 8th edition, 2007: Elsevier. – 1196 p.

4. Disorders of purine metabolism and gouty nephropaty [E-resource]. – Access mode: URL: http://www.lvrach.ru/2006/10/4534541/.

5. Dzhonnazarova D. Clinical and renal function in gout among residents of the Republic of Tajikistan. Abstract, 2013

6. Lukyanchuk E. Experience of the use of nimesulide for the relief of pain in gouty arthritis. The Ukrainian journal of rheumatology. No. 1 (51), 2013, 53–55.

7. European Scientific Cooperative on Phytotherapy. Centaurii herba (Centaury herb). In: ESCOP Monographs. 2nd ed. Stuttgart, Germany, and New York: Thieme-Verlag, 2003: 70–73.

8. European Scientific Cooperative on Phytotherapy. Rosmarini folium (Rosemary leaves). In: ESCOP Monographs. 2nd ed. Stuttgart, Germany, and New York: Thieme-Verlag; 2003: 429–436.

9. Gaybullaev AA, Kariev SS. Effects of the herbal combination Canephron_N on urinary risk factors of idiopathic calcium urolithiasis in an open study. Z Phytother 2013; 34: 16–20.

10. Gerald D, Nazia R, Fang N. Effect of Urate-lowering Therapies on Renal Disease Progression in Patients with Hyperuricemia. The journal of Rheumatology, 2014. 955–962

11. Gracza L, Koch H, Löffler E. Isolierung von Rosmarinsäure aus Symphytum officinale und ihre antiinflammatorische Wirksamkeit in einem In-vitro. Modell. Arch Pharm 1985; 318:1090–1095.

12. Haloui M, Louedec L, Michel B, Lyoussi B. Experimental diuretic effects of osmarinus officinalis and Centaurium erythraea. J Ethnopharmacol 2000; 71: 465–472.

13. Hossamel Z, Brian F. Managing gout: How is it different in patients with chronic kidney disease?

Cleveland clinical journal of medicine volume 77 / Number 12, 2010, 919–928.

14. Hovind P. Serum uric acid as a predictor for development of diabetic nephropathy in type 1 diabetes. An inception cohort study. Diabetes. 2009. – V. 58. 1668–1671.

15. Jalal D. Serum uric acid levels predict the development of albuminuria over 6 years in patients with type 1 diabetes: findings from the coronary artery calcification in type 1 diabetes study. Nephrol Dial Transplant. 2010. N25. 1865–1869.

16. Kanbay M, Solak Y, Dogan E et al. Uric acid in hypertension and renal disease: The chicken or the egg? Blood Purif, 2010. – N 30. 288–295.

17. Kumarasamy Y, Nahar L, Cox PJ, et al. Bioactivity of secoiridoid glycosides from Centaurium erythraea. Phytomedicine 2003; 10: 344–347.

18. Kumarasamy Y, Nahar L, Sarker SD. Bioactivity of gentiopicroside from the aerial parts of Centaurium erythraea. Fitoterapia 2003; 74: 151–154.

19. Martynyuk L, Martynyuk L, Ruzhitska O, Martynyuk O. Effect of the Herbal Combination Canephron N on Diabetic Nephropathy in Patients with Diabetes Mellitus: Results of a Comparative Cohort Study. The journal of alternative and complementary medicine, 2014, 1–7.

20. Microalbuminuria [E-resource]. – Access mode: URL: http://www.indap.info/mikroalbuminuriya.html.

21. Naber KG. Efficacy and safety of the phytotherapeutic drug Canephron_N in prevention and treatment of urogenital and gestational disease: review of clinical experience in Eastern Europe and Central Asia. Res Rep Urol 2013; 5: 39–46.

22. Novitsky V. Pathophysiology. Edited. Vladimir Novitsky, ED Goldberg, OI Urazova. – M .: GEOTAR Media, 2009. – T. 2. – with 848.

23. Rampart M, Beetjens JR, Bult H, et al. Complementdependent stimulation of prostacyclin biosynthesis; inhibition by rosmarinic acid. Biochem Pharmacol 1986; 35: 1397–1400.

24. Richard J, Takahiko N, Diana J, Laura G et al. Uric acid and chronic kidney disease: which is chasing which? Nephrology Dialysis Transplantation, Volume 28, Issue 9. 2221–2228.

25. Shuba N, Voronov T, Tkachenko N. Influence of complex phytodrug Canephron N on the level of uric acid in patients with hyperuricemia and arterial hypertension. ML №1 (77) 2011. 77–79.

26. Sterner W, Heisler E, Popp HO, Fischer H. Studien über die Canephron-Wirkung bei chronischen Nierenerkrankungen. Physikalische Medizin Rehabilitation 1973; 14: 239–258.

27. Testa A, Mallamaci F, Spoto B et al. Association of a Polymorphism in a Gene Encoding a Urate Transporter with CKD Progression. Clinical Journal of the American Society of Nephrology, 2014. 1059–1065.

28. The study of renal function [E-resource]. – Access mode: URL: http://www.biochemmack.ru/upload/uf/204/2042dda688fc61b5d5db08ad2350d6dc.pdf.

29. Valentao P, Fernandes E, Carvalho F, et al. Hydroxyl radical and hypochlorous acid scavenging activity of small centaury (Centaurium erythraea) infusion. A comparative study with green tea (Camellia sinensis). Phytomedicine 2003;10: 517–522.

30. Viazzi F, Leoncini G, Ratto E et al. Mild hyperuricemia and subclinical renal damage in untreated primary hypertension. AJH, 2007. V. 20; 1276–1282.

31. Weiner D. Uric acid and incident kidney disease in the community. J Am Soc Nephrol, 2008. – V. 19, N 6. 1204–1211.

32. Yamahara J, Konoshima I, Sawada I, Fujimura H. Biologically active principles of crude drugs: pharmacological actions of Swertia japonica extracts, swertiamarine and gentianine. Yakugaku Zasshi 1978; 98: 1446–1451.

33. Yarnell E. Botanical medicines for the urinary tract. World J Urol 2002; 20: 285–293.

Received: 2016-02-10

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 9–12

DOI 10.11603/ijmmr.2413-6077.2016.1.6372

COMPLEX APPROACH TO TREATMENT OF SUBCHORIONIC HEMATOMA IN EARLY THREATENED ABORTION

S. N. Heryak, N. V. Petrenko, I. Ya. Kuziv, O. Y. Stelmakh, N. I. Bagniy, I. V. Korda, V. Yu. Dobryanska, L. V. Bagniy
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

Background. *Currently, miscarriage is considered to be a multietiological disorder with trombofilic violations and hormone deficiency as the leading factors. Despite the achievements in treatment of miscarriage, the frequency of preterm termination of the wanted pregnancies is still high and the number of perinatal losses is significant. Therefore, pathogenetically based therapy, safe for the foetus, is very important in management of pregnancy interruption in the first trimester. A proper drugs administration provides optimal concentration of active ingredients and fast action. The aim is to improve effectiveness of the early threatened abortion treatment in cases of subchorionic hematoma (SCH) by combination of sublingual natural micronized progesterone and tranexamic acid*

Objective. *We examined 50 pregnant women with early threatened abortion with SCH. We studied system of haemostasis, basic hormonal markers and ultrasound criteria of threatened abortion. We compared efficacy of treatment between traditional (supportive) therapy (sedation, spasmolytic, haemostatic drug) and combination of supportive therapy in combination with tranexamic acid and natural micronized progesterone.*

Results. *The result of lab tests showed minimal signs of hypercoagulation, hyperfibrinogenemia and platelet hyperactivity, a significant β-hCG level decrease and approximate decrease in progesterone and free estriol production.*

Sonographic examination showed presents of local myometrial hypertonus, deformation of fertilized egg, hypoplasia of chorion, low location of fertilized ovum, retarded growth of CRL.

The research proved that combined administration of sublingual micronized progesterone and tranexamic acid for the treatment of threatened abortion with SCH has more significant positive effect for pregnancy maintenance due to clinical, biochemical, hormonal and ultrasound results if compared with the group which underwent supportive therapy.

Conclusions. *Complex application of natural micronized progesterone 100 mg three times a day sublingually and 500 mg of Tranexamic acid dissolved in 200 ml normal saline solution improves the dynamics of the main hormonal, haemostatic and ultrasound markers of abortion and significantly reduces reproductive losses. Tranexamic acid treatment proved a rapid and effective action on hematoma and absence of embryotoxical and coagulopathyc influence. Tranexamic acid does not cause any significant disorders of hemostatic system. This is very important at the early gestation because of intravascular coagulation, physiological hypercoagulable condition during pregnancy that can cause microthrombosis and disrupt placentation. On the other hand, it is dangerous for the mother's health because of the increased risk of thrombosis.*

KEY WORDS: **threatened miscarriage, subhorial hematoma, micronized progesterone, tranexamic acid.**

Introduction

Currently, miscarriage is considered to be a multietiological disorder with trombofilic violations and hormone deficiency as the leading factors. The problem of early pregnancy loss remains urgent because these factors are the most common complications without any downward tendency [1–4]. According to the latest concepts, 80% of all pregnancy losses occur at the early stages. One of the first signs that threatens the pregnancy is subchorionic hematoma (SCH) [5, 6]. It is the most unfortunate sign of threatened abortion. It is established that the disruption of trophoblast invasion in the 1st trimester of pregnancy leads to the advanced gestational complications: threatened abortion, preeclampsia, preterm delivery, placental abruption, which increase perinatal, infant and

Corresponding author: Svitlana Heryak, Department of Obstetrics and Gynecology No 2, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel.: +380352254891
E-mail: heryak@tdmu.edu.ua

maternal mortality. The protective function of the immune system at the early stages of gestation such as the suppression of natural killer cells in the endometrium, mother's blocking factors production is provided by different mostly hormonal dependent mechanisms; their disorders lead to defects of implantation, SCH and can cause early pregnancy termination [3, 7].

The endometrium and decidua contain a huge number of immune system cells, all of them are able to secrete cytokines. Main cytokines that are secreted in the body inhibit the embryo development, proliferation and development of normal trophoblast. They affect the embryo, both directly and indirectly, depending on the intensity of secretion and differentiation of target tissues [6]. Cytokine cascade can be triggered by infectious agent and by endogenous factors (hypoxia, hormones, etc.) as well. Persistent viral bacterial infection, neuroendocrine disorders, chromosomal abnormalities, the toxic effects, stress and environment are also etiological factors of early abortion [8, 9]. Despite the achievements in treatment of miscarriage, the frequency of preterm termination of the wanted pregnancies is still high and the number of perinatal losses is significant. Therefore, pathogenetically based therapy, safe for the foetus, is very important in management of pregnancy interruption in the first trimester. A proper drugs administration provides optimal concentration of active ingredients and fast action.

The aim is to improve effectiveness of the early threatened abortion treatment in cases of subchorionic hematoma by combination of sublingual natural micronized progesterone and tranexamic acid.

Materials and Methods

The examination of 50 pregnant women was conducted at the Department of Gynaecology of Ternopil Regional Perinatal Centre "Mother and Child". They received treatment for threatened abortion in gestational age from 8 to 12 weeks. All women were diagnosed with SCH by means of ultrasound.

To exclude infection as an etiological factor of abortion, bacteriological research of TORCH and sexually transmitted infections were conducted. All patients had negative results of the tests for these infections.

System of haemostasis was evaluated by determination of platelet and aggregation time, prothrombin index (PI), thrombin clotting time, activated partial thromboplastin time (APTT), fibrinogen concentration.

Basic hormonal markers of abortion were also studied: the concentration of free estriol and progesterone by radioimmunoassay method, sets made by company "SORIN" (France), and radioisotope sets Amerlayt made by international company Amersham. β-subunit of human chorionic gonadotropin (β-hCG) concentration was determined by immune fluorescence analysis with time resolution (test system Delfiya, Wallac, Perkinelmer) (according to the manufacture standard protocol of test systems).

To determine efficacy of the proposed complex of therapeutic measures, all the pregnant were tested with transvaginal ultrasound on the 14th day of the treatment.

The pregnant of the 1st group (n=25; the comparison group) received supportive therapy – sedation, spasmolytic, haemostatic drug. The pregnant of 2nd group (n=25), together with supportive therapy, received tranexamic acid 2 ml (500 mg) dissolved in 200 ml normal saline solution intravenously till the arrest of bleeding and natural micronized progesterone 100 mg three times a day sublingually. The control group consisted of 20 healthy pregnant women without any symptoms of threatened abortion.

Results and Discussion

The evaluation of plasma-coagulation and vascular-platelet hemostasis showed minimal signs of hypercoagulation, hyperfibrinogenemia and platelet hyperactivity if compared with the women with physiological pregnancy. This suggests a thrombophilia as an etiopathogenetic factor of early abortion with SCH in the examined women.

The evaluation of hormonal homeostasis showed, that at the early gestational age a significant β-hCG level decrease and approximate decrease in progesterone and free estriol production were the diagnostic markers of threatened abortion with SCH

The results of ultrasound examination showed, that SCH was located in the area of the lower chorion in 32 (64%) of the pregnant women, in the central part – in 18 (36%) patients. Also sonographic examination showed other markers of abortion. So, local myometrial hypertonus was diagnosed in 43 (86%) pregnant women, deformation of fertilized egg – in 32 (64%), hypoplasia of chorion – in 12 (24%), low location of fertilized ovum – 8 (16%), retarded growth of CRL – in 2 (4%).

The research proved that combined administration of sublingual micronized progesterone

and tranexamic acid for the treatment of threatened abortion with SCH has more significant positive effect for pregnancy maintenance due to clinical, biochemical, hormonal and ultrasound results if compared with the group which underwent supportive therapy.

After the symptomatic treatment a spontaneous abortion happened in 6 (24%) women, missed abortion in – 6 (24%), gestational process complicated by hyperemesis gravidarum which required additional therapy – in 4 (16%). Among 25 women of the 2nd group spontaneous abortion occurred in 3 (12%) patients, missed abortion – in 1 (4%) woman.

The results of our research have shown that in women of the 2nd group the level of platelets increased and did not significantly differ from the control group findings. In patients of the 1st group it was significantly lower than in women of the 2nd and control groups. The similar changes were evidenced in the evaluation of PI.

In 2 weeks of the proposed therapy, the level of β-hCG increased in both groups of women, but in the 2nd group it did not significantly differ from the control group. After the treatment the level of free estriol and progesterone increased and did not significantly differ from the control group with the similar results.

Symptomatic and complex treatment significantly reduced the sonographic signs of abortion, but in the 2nd group these changes pronounced more positive trend than in the 1st group. So, in 18 (72%) pregnant women of the 2nd group and in 4 (16%) – of the I group ($p<0,05$) there was a complete resumption of SCH, hematoma decreased in size in 3 (12%) women of each group.

Thus, the prescription of micronized progesterone sublingually provides an inhibitory effect on the contractile activity of myofibrils, prevents the further chorine detachment that leads to pregnancy maintenance and prevents reproductive losses.

Our research demonstrates the necessity of the inclusion of micronized progesterone in 100 mg 3 times daily sublingually and tranexamic acid 500 mg intravenously to the treatment protocol of early threatened abortion with SCH. Also this treatment is more effective if compared with the traditional supportive therapy (sedation, spasmolytic, haemostatic drug).

Conclusions

1. Complex application of natural micronized progesterone 100 mg three times a day sublingually and 500 mg of Tranexamic acid dissolved in 200 ml normal saline solution improves the dynamics of the main hormonal, haemostatic and ultrasound markers of abortion and significantly reduces reproductive losses.

2. Tranexamic acid treatment proved a rapid and effective action on hematoma and absence of embryotoxical and coagulopathyc influence. Tranexamic acid does not cause any significant disorders of hemostatic system. This is very important at the early gestation because of intravascular coagulation, physiological hypercoagulable condition during pregnancy that can cause microthrombosis and disrupt placentation. On the other hand, it is dangerous for the mother's health because of the increased risk of thrombosis.

References

1. Венцківський БМ. Стан імунного та гормонального статусусфетоплацентаного комплексу при недоношуванні вагітності. Педіатрія, акушерство та гінекологія 2012; 3: 40-43.

2. Резніченко ГІ. Профілактика невиношування вагітності і передчасних пологів. Жіночий лікар 2013; 3: 10-12.

3. Soldo V, Cutura N, Zamurovic M. Threatened miscarriage in the first trimester and retrochorial hematomas: sonographic evaluation and significance. Clin Exp Obstet Gynecol 2013; 40(4): 548-50.

4. Şukur YE, Goç G, Kose O, Acmaz G, Ozmen B, Atabekoglu CS, Koc A, Soylemez F. The effects of subchorionic hematoma on pregnancy outcome in patients with threatened abortion. J Turk Ger Gynecol Assoc 2014; 15(4): 239-242.

5. Dongol A, Mool S, Tiwari P. Outcome of pregnancy complicated by threatened abortion. Kathmandu Univ Med J 2011; 9(33): 41-44.

6. Odeh M, Ophir E, Grinin V, Tendler R, Kais M, Bornstein J. Prediction of abortion using three-dimensional ultrasound volumetry of the gestational sac and the amniotic sac in threatened abortion. J Clin Ultrasound 2012; 40(7): 389-393.

7. Biesiada L, Krekora M, Krasomski G. Subchorionic hematomaas a risk factor of pregnancy and

delivery in women with threatening abortion. Ginekol Pol 2010; 81(12): 902-906.

8. Hodgson DT, Lotfipour S, Fox JC. Vaginal bleeding before 20 weeks gestation due to placental abruption leading to disseminated intravascular coagulation and fetal loss after appearing to satisfy criteria for routine threatened abortion: a case report and brief review of the literature. J Emerg Med 2009; 32(4): 387-392.

9. Stamatopoulos N, Lu C, Infante F, Menakaya U, Casikar I, Reid S, Mongelli M, Condous G. Does the presence of subchorionic haematoma increase the risk of miscarriage? Ultrasound in Obstetrics & Gynecology 2013; 42 (1): 54.

Received: 2015-12-01

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 13-18

DOI 10.11603/ijmmr.2413-6077.2016.1.6373

DETECTION OF OXIDATIVE STRESS, APOPTOSIS AND MOLECULAR LESIONS IN HUMAN OVARIAN CANCER CELLS

H. I. Falfushynska[1,2,3]
[1]*I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE*
[2]*VOLODYMYR HNATIUK TERNOPIL NATIONAL PEDAGOGICAL UNIVERSITY, TERNOPIL, UKRAINE*
[3]*UNIVERSITY OF NORTH CAROLINA AT CHARLOTTE, CHARLOTTE, USA*

Background. *Ovarian cancer has the highest mortality rate of gynaecological cancers. This is partly due to the lack of effective screening markers. Indices of oxidative stress are well-recognized prognostic criteria for tumorous transformation of tissue, but their value depends on the type of tumor and the stage of its development.*

Objective. *The aim of this study is to clarify the relationship between antioxidant/pro-oxidant ratio and the signs of molecular lesions and apoptosis rate in blood of ovarian cancer patients and non-cancer ones.*

Results. *The ovarian cancer group is marked by antioxidant/prooxidant balance shifting to oxidative damage in blood as the consequence of overexpression of oxyradicals (by 300%). Higher level of glutathione (by 366%), lower level of metallothioneins (by 65%) as well as higher level of lipid peroxidation (by 174%) and protein carbonyls (by 186%) in blood of ovarian cancer patients compared to the normal ovarian group have been observed. The signs of cytotoxicity are determined in blood of ovarian cancer patients: an increased (compared to control) level of DNA fragmentation (by 160%), choline esterase (up to twice), higher rate of both caspase dependent and caspase independent lysosomal mediated apoptosis.*

Conclusions. *Cathepsin D activity both total and free, choline esterase activity, TBA-reactive substance and protein carbonyls level in blood could be used as the predictive markers of worse prognosis and the signs of human ovarian cancer.*

KEY WORDS: **ovarian cancer, oxidative stress, apoptosis, caspase-3, cathepsin D, choline esterase, metallothionein.**

Introduction

Ovarian cancer dominates among the death causes of malignant tumours. In particular, according to the International Agency for Cancer Research, more than 165 thousand of newly diagnosed cases of ovarian cancer are registered each year over the world. Despite intensive studies, every year, more than 100 000 women die from this disease worldwide. Due to minimal and non-specific early stage symptoms, ovarian cancer diagnosis is late and prognosis is usually poor [1]. Currently there are no screening programs for precancerous and malignant ovarian pathology diagnostic, with the help of which specialists could have reduced the incidence and fatalities of this disease [2]. Therefore, exploration of new and/or low-cost early-warning molecular signatures of disease is a key goal of ovarian cancer research.

One of the most common mechanisms of diseases development and aggressiveness of pathology which is on the focus of biomedical research has been linked to overexpression of free radicals and oxidative stress initiation [3, 4, 5, 6, 7]. The damage that reactive oxygen species (ROS) can cause to the cell not only depends on their internal concentration but also on the equilibrium between the ROS and the endogenous antioxidants normally protect cells against oxidative stress [4, 6]. When the antioxidant/prooxidant system becomes unbalanced, oxidative stress is generated, altering and damaging many biomolecules, including DNA, lipids and proteins, in turn, decreasing cell viability [5, 6, 8, 9]. We have shown before that higher content of the products of oxidative damage of proteins, lipids, DNA fragmentation and cathepsin D activity, particularly free one are the main characteristic signs of malignant ovarian tissue [10]. However, it can be difficult

Corresponding author: Halina Falfushynska, Department of General Chemistry, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Research Laboratory of Comparative Biochemistry and Molecular Biology, Volodymyr Hnatiuk Ternopil National Pedagogical University, 2 Maksym. Kryvonis Street, Ternopil, Ukraine, 46027
Tel.: +380673506531
E-mail: halynka.f@gmail.com

to obtain a biopsy from ovarian tissue for routine biochemical measurements with prognostic mission of diseases development. Instead of them correspondent blood test would be highly recommended. Thus, here we aim to clarify the relationship between antioxidant/pro-oxidant ratio and the signs of molecular lesions and apoptosis rate in blood of ovarian cancer patients and normal ovarian ones.

Materials and Methods

For this research we used the venous blood samples taken from the 15 newly diagnosed patients of reproductive age who had been operated for epithelial ovarian cancer at the Department of Gynaecology of Ternopil Regional Oncological Hospital. Cancer pathology was verified histologically. According to FIGO classification, all diagnosed patients had stage III disease. None of the operated oncologic female patients had been previously treated with platinum-based drugs (cisplatin/carboplatin/cycloplatam) or cyclophosphamide. Venous blood of 15 females not affected by the relevant pathology were taken as controls. All experimental studies were conducted in accordance with the rules of the National Congress on Bioethics (Kyiv, 2000) and the decision of the Commission on Bioethics of the Ternopil State Medical University (№ 3, 2013).

All the procedures on bloods were carried out at 4° C. All the reagents, except those specified below, were produced by "Synbias", 'chemically pure' grade.

The determination was conducted in the soluble fraction of blood, which had been received to determine superoxide dismutase (SOD) as a result of centrifugation of blood within 10 min at 6000 *g* (S6), to determine the level of oxygen radicals – as a result of its centrifugation within 45 min at 12 000 *g* (S12) and to determine the concentration of metallothioneins – within 45 min at 16 000 *g* (S16).

Superoxide dismutase activity (SOD) (EC 1.15.1.1) was measured by a decrease in the rate of the reduction of Nitrotetrazolium blue in the presence of phenazine methosulfate and NADH [11]. Enzymatic activity was expressed in conventional units (*CU*). Enzyme activity, which was able to cause a decrease in optical density in the process of the reduction of Nitrotetrazolium blue in 50% test sample per 1 mg of protein from the homogenate in soluble form, was taken as 1 *CU*.

The content of total glutathione was determined in blood after complete reduction of glutathione through the use of glutathione reductase (*Sigma*, USA) and with the help of Ellman's reagent [12]. The level of 5-trinitrobenzoic acid was monitored with a spectrophotometer at 412 nm.

The determination of protein carbonyls (PC) was conducted due to their ability to form 2,4-dinitrophenylhydrazones under the blood plasma incubation in the presence of 0.1 M 2,4-dinitrophenylhydrazine in HCl, 2 M. The light absorbance was registered at 370 nm against the control, and the content of phenylhydrazone was calculated using a molar extinction coefficient of 2.1×10^4 $M^{-1}cm^{-1}$[13]. Lipid peroxidation was characterized by the products of interaction between deproteinized blood after precipitation of proteins with trichloroacetic acid (with final concentration of 5%) from blood samples and 2-thiobarbituric acid (TBA). The formation of TBA-reactive substance (TBARS) was calculated by the intensity of the absorption of a pink-coloured complex at 532 nm by the molar extinction coefficient of the complex equal to $\varepsilon=1.56\times10^5$ $M^{-1}cm^{-1}$ [14].

The content of oxyradicals (OR) in S12 fraction of the blood plasma was evaluated using the non-fluorescent derivative, dihydrorhodamine, which is converted to the fluorescent dye, rhodamine-123, after a reaction with reactive oxygen species. The fluorescence signal was detected by using an *f*-max fluorescence plate-reader [excitation=485 nm, emission=538 nm] immediately and after 20 min and used to determine the rate of ROS formation [10, 15] and expressed in relative fluorescence units (RFU) per 1 mg of protein.

The content of metallothioneins (MTs) in S16 fraction of blood was evaluated by the content of thiol groups (MT-SH) with slight modification [8, 9, 10, 16] with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB, *Sigma*, USA) after the chloroform-ethanol extraction of MT, and calculated assuming that 1 mol of MT contains the same amount of SH-groups as 20 moles of GSH.

Choline esterase (ChE, EC 3.1.1.7) activity was determined in blood as the acetylthiocholine-cleaving ChE activity according to the colorimetric method of Ellman et al. (1961) at 25°C [17]. Enzyme activity was calculated using a molar extinction coefficient of 13.6×10^3 $M^{-1}cm^{-1}$ and standardized to the soluble protein content.

DNA damage was determined by the content of the fragmented deproteinized DNA in the total DNA by the method of alkaline pre-

cipitation of 10% blood plasma in 50 mM Tris-EDTA buffer, pH 8.0, containing 0.5% sodium dodecyl sulfate (*Sigma*, USA). The supernatant contains damaged DNA molecules when the pellet contains protein and a whole DNA. DNA content in the supernatant and in the pellet was determined by the Hoescht dye in the presence of 0.4 M NaCl, 4 mM of sodium cholate and 0.1 M Tris (pH 9.0) at the excitation wave (ex.)=360 nm and emission (em.)=450 nm [18]. The content of fragmented DNA was expressed as a percentage to the total DNA in the sample.

Caspase-3 activity in blood samples was measured using a colorimetric assay based on the hydrolysis of peptide acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in a release of the p-nitroaniline (pNA) moiety. p-Nitroaniline was detected at 405 nm (ε_M=10,500 $M^{-1}cm^{-1}$) [19, 20].

The enzymatic activity of cathepsin D (EC 3.4.23.5) was determined spectrophotometrically by the formation of acid-soluble products of hemoglobin enzymatic hydrolysis [10, 21]. The reaction mixture contained 50% blood serum in 0.25 M of sucrose solution and, as a substrate, 1% solution of bovine hemoglobin (*Sigma*, USA) in 0.1 M acetate buffer (pH 5.0). The enzymatic reaction was stopped by adding 10% solution of trichloracetic acid up to a final concentration of 2%. To determine the total activity of cathepsin D, the blood serum sample was previously treated with 1% solution of Triton X-100 (*Sigma*, USA) for 10 min at +37°C. The control sample was incubated at +4°C for 30 min before adding 10% trichloracetic acid solution. The activity of cathepsin D was calculated by the difference in the optical density of experimental and control samples at 280 nm wave length and expressed as nmol of tyrosine/(min×mL).

The results of the measurements were presented as means ± standard deviation (SD) M±SD for 15 samples of the blood of the ovarian cancer patients and the reference group. If the data was not normally distributed according to the Lilieford test, it had been transformed using the Box-Cox method. For the data that were not normally distributed even after the transformation, non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney U-test) were performed. Differences were considered significant if the probability of Type I error was less than 0.05. For evaluation of the antioxidative-prooxidative equilibrium we proposed the Integrated Oxidative Stress (IOS) index as the ratio of antioxidant factors (SOD, GSH and MT-SH level) and prooxidant manifestations (oxyradicals (OR), TBARS and PC) after the standardization of data [8, 9]. The main classification criterion and relationship between biochemical parameters of the ovarian tissue samples was evaluated using the discriminant analysis and correlation analysis (Pearson's correlation coefficient *r* under the probability of the value p<0.05). All statistical calculations were performed by means of Statistica v10.0 and Excel for Windows 2010.

Results and Discussion

The results of an evaluation of antioxidant defense system parameters (Table 1) show glutathione level (by 366%) in blood of the cancer patients is higher than in the control ones and SOD activity just commensurate in both groups. The MT-SH level (by 65%) is lower under the ovarian cancer. At the same time, the intensity of the oxyradicals formation (by 300%), the concentration of TBARS (by 174%) and protein carbonyls (by 186%) are higher in the blood of the cancer patients compared to the correspondent control ones.

The signs of cytotoxicity are determined in blood of the ovarian cancer patients: an increased (compared to the control ones) level of DNA fragmentation (by 160%), caspase-3 activity (by 43%) and cathepsin D activity, both of its total (by 546%) and, particularly, free forms (by 952%). The activity of choline esterase is twice as high as in the ovarian cancer group compared to the intact one.

The application of discriminant functional analysis (a subtype of the multivariate data analysis) to the set of studied biochemical parameters has shown that the characteristics of cathepsin D activity both total and free, choline estrase activity, TBARS and protein carbonyls level are the main criteria for differentiation of the studied groups (F(11,18)=2353,6, p<0,0000). For an appreciation of the quantitative equilibrium between antioxidant defence and oxidative destruction, we calculated the IOS index after standardizing the data. It was lower in blood of the cancer patients (IOS=-39%) indicating the balance shifting to oxidative damage as the consequence of tolerance limits of antioxidant defence system increased in ovarian cancer patient.

Recent studies have shown an important role for reactive oxygen species (ROS) in cancer development [5]. ROS can be produced in different endogenous sources, such as mitochondria, peroxisomes etc. [3] and are accumulated

Table 1. Biochemical parameters in blood of ovarian cancer and normal ovarian patients, M±SD, n=15

Parameter	Groups	
	Normal ovarian	Ovarian cancer
MT-SH content, μg·mL^{-1} of blood	16.2±1.9	5.6±0.7*
Total glutathione content, mmol·L^{-1} of blood	0.9±0.1	4.2±0.8*
SOD activity, CU·mg^{-1} of protein	1.8±0.2	1.6±0.2
TBARS content, nmol·mL^{-1} of blood	19.4±2.5	53.1±6.2*
Protein carbonyls level, nmol·mg^{-1} of plasma proteins	0.7±0.2	2.0±0.3*
Cholin esterase, nmol·min^{-1}·mg^{-1} proteins	0.38±0.04	1.25±0.18*
Oxygen radicals content, RFU·mg^{-1} of protein	0.94±0.08	3.76±0.42*
Caspase-3, pmol·min^{-1}·mg^{-1} proteins	7.7±0.8	11.0±1.3*
Total activity of cathepsin D, nmol·min^{-1}·mL^{-1} of blood serum	2.2±0.3	14.2±1.2*
Free cathepsin D activity, nmol·min^{-1}·mL^{-1} of blood serum	0.96±0.07	10.10±0.89*
Content of fragmented DNA in total DNA, %	3.0±0.4	7.8±0.5*

Note. * – differences compared to the blood samples of the reference group, $p<0.05$.

under an imbalance between their production and elimination by protective mechanisms, referred to as antioxidants. ROS are overproduced a long time, then, may cause DNA, protein, and/or lipid damage [7]. In our case the ovary cancer is also said to be under oxidative stress (IOS less than zero) associates with the most prominent upraise of oxidative lesions (triple of them, TBARS, protein carbonyls and oxyradicals). We speculate that oxidative stress causes injury to cells, among them initiation of DNA strand breaks (DNAsb=1.65–0.02×TBARS+ 1.93×PC*+0.65×OR*, R^2=0.98, F(6,23)=212,69, p<0.001; * – parameter makes a presumable contribution to the mathematical model) and liberation of cathepsin D from lysosomes (Cathepsin D(free) = –3.69+0.19×TBARS*+0.78× PC+0.48×OR, R^2=0.96, F(6,23)=210,05, p<0.001). It is established that cathepsins, released from lysosomes in course of oxidative injury of membranes [22], cleave Bid, which activates the mitochondrial apoptosis pathway [23]. Thus, human ovary cancer progression is linked to oxidative stress directly by increasing DNA fragmentation and caspase-independent lysosomal-mediated apoptosis pathway.

A failure to undergo apoptosis is considered to be a key event in cancer formation and progression [24]. Using the key mediators of mitochondrial events of *apoptosis*, caspase 3, we have shown that ovarian cancer exhibits higher rates of apoptosis than either samples of the control group. Data about caspase 3 role in cancer progress and patients survival are controversial; and it has been demonstrated that expression of caspase-3 does not correlate with the extent of apoptosis in primary carcinomas [25]. Some investigators have also reported increased rates of apoptosis in different types of cancer, breast carcinomas among them. But, on the other hand, it has been shown that loss of caspase-3 expression may decrease breast cancer cells resistant to chemotherapy and radiation therapy [26].

Many studies have found that the expression level of AChE and/or BChE increases during apoptosis in various cell types and alter human breast, lung cancer, leukemia etc progress [27]. Many tumours, including ovarian carcinoma, express ChEs, indicating that the enzymes may be functionally important in neoplastic cell transformation, and can be considered a marker of early differentiation [28]. In breast cancer AChE activity is as in twice as high as in normal breast. The difference in lectin reactivity between erythrocyte and breast AChE, the lack of AChE in blood plasma suggest that breast epithelial cells produce AChE for membrane attachment [27]. It has also been demonstrated that AChE can hydrolyse lipid peroxides, which may support the possibility that a reduction in enzyme activity augments oxidative stress and cellular damage of bronchopulmonary epithelial cells [29]. We have also shown in our study the concordant changes of choline esterase and caspase-3 activity (r=0.83, p<0.001) strongly suggest that ChE is an important component in the common pathway leading to apoptosis in human ovarian cancer and future studies of such mechanisms would be of great interest.

Several *in vitro* studies have demonstrated the importance of MTs expression for cancer cell growth and survival. On the one hand overexpression of MTs has been reported for different type of cancers and MT levels have been correlated with increasing tumour grade in different ones [30, 31]. But, on the other hand, the downregulation of MT protein production

resulted in dramatic growth inhibition of several human cancer cell lines. Similarly, when MT expression was abolished in human PC-3 prostate and SKOV-3 ovarian cancer cell lines using a specific ribozyme, there is a marked increase in spontaneous apoptosis [32]. We have also found that lower level of MTs in ovarian cancer group potentially caused the caspase-dependent apoptosis pathway activation ($r=-0.86$, $p<0.001$). We speculate that apoptotic activation is dependent on MTs levels via zinc, because it is a potent inhibitor of caspase-3 [33]. It is recognised that depletion of zinc by chelation has been shown to promote apoptosis *in vitro* [34]. Therefore, zinc bound to MTs may play a crucial role in the control of apoptosis. Apoptosis rate would be as higher as less zinc is liberated in cells containing less MTs. Our present findings are in good correlation with our previous work that demonstrated decreased zinc concentration in the samples of ovarian cancer tissue compared with normal ovarian [10].

Conclusions

Using the multi-marker analysis of stress-related processes in the ovarian cancer we determined a number of blood characteristics, which compound the pathological changes in the malignant tissue; the imbalance between antioxidants and oxidative lesions, appearance of DNA strand breaks and activation of both caspase dependent and caspase independent lysosomal mediated apoptosis pathway are among them. Cathepsin D activity, both total and free, choline esterase activity, TBARS and protein carbonyls level can be used as the predictive markers of worse prognosis and the signs of human ovarian cancer. Metallothioneins down-regulation and choline esterase activation in blood are ultimately resulting in intrinsic apoptosis pathway in ovarian cancer. The further studies are needed to elucidate the mechanism by which choline esterase regulates caspase-3 dependent apoptosis in human ovarian cancer.

Acknowledgements

The research was supported by the West-Ukrainian Biomedical Center, the Ministry of Education and Science of Ukraine (State Budget Topic № 125B) and Fulbright Scholar grant.

References

1. Howlader N, Noone AM, Krapcho M, Garshell J, Neyman N, Altekruse SF et al. SEER Cancer Statistics Review, 1975-2010, National Cancer Institute. Bethesda, MD, http://seer.cancer.gov/csr/1975_2010/, based on November 2012 SEER data submission, posted to the SEER web site (accessed, April 2013). Controlled Trial. JAMA 2011; 305: 2295–2303.

2. Klaunig JE, Kamendulis LM, Hocevar BA. Oxidative stress and oxidative damage in carcinogenesis. Toxicol Pathol 2010; 38: 96–109.

3. Mahalingaiah PKS, Singh KP. Chronic oxidative stress increases growth and tumorigenic potential of MCF-7 breast cancer cells. PLOS ONE 2014; 9: e93799.

4. Reuter S, Gupta SC, Chaturvedi MM, Aggarwal BB. Oxidative stress, inflammation, and cancer: How are they linked? Free Radic Biol Med 2010; 49: 1603–1616.

5. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact 2006; 160: 1–40.

6. Visconti R, Grieco D. New insights on oxidative stress in cancer. Curr Opin Drug Discov Devel 2009; 12: 240–245.

7. Falfushynska H, Gnatyshyna L, Shulgai A, Shidlovsky V, Stoliar O. Oxidative stress in human thyroid gland in cases of iodine deficiency nodular goitre: from harmlessness to hazard depending on copper and iodine subcellular distribution. Int J Med Medical Res 2015; 1: 5–11.

8. Falfushynska HI, Gnatyshyna LL, Osadchuk OY, Shidlovsky VO, Stoliar OB. Trace elements storage and metallothioneins function in cases if human thyroid gland transformation. Ukr Biochem J 2014; 86: 107–113.

9. Falfushynska HI, Gnatyshyna LL, Deneha HV, Osadchuk OY, Stoliar OB. Manifestations of oxidative stress and molecular damages in ovarian cancer tissue. Ukr Biochem J 2015; 87: 93–102.

10. Beauchamp C, Fridovich I. Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. Anal Biochem 1971; 44: 276–287.

11. Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Meth Enzymol 1985; 113: 548–555.

12. Lushchak VI, Bagnyukova TV, Lushchak OV. Indices of oxidative stress. 1. TBA-reactive substances and carbonylproteins. Ukr Biochem J 2004; 76: 136–141.

13. Ohkawa H, Ohishi N, Tagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351–358.

14. Viarengo A, Burlando B, Cavaletto M, Marchi B, Ponzano E, Blasco J. Role of metallothionein against oxidative stress in the mussel *Mytilus galloprovincialis*. *Am J Physiol* 1999; 277: 1612–1619.

15. Viarengo A, Ponzano E, Dondero F, Fabbri R. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Mar Environ Res 1997; 44: 69–84.

16. Ellman GL, Courtney KD, Andres VJ, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961; 7: 88–95.

17. Olive PL. DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. Environ Mol Mutagen 1988; 11: 487–495.

18. Bonomini M, Dottori S, Amoroso A, Arduini A, Sirolli V. Increased platelet phosphatidylserine exposure and caspase activation in chronic uremia. J Thromb Haemost 2004; 2: 1275–1281.

19. Falfushynska H, Gnatyshyna L, Fedoruk O, Mitina N, Zaichenko A, Stoliar O, Stoika R. Hepatic metallothioneins in molecular responses to cobalt, zinc, and their nanoscale polymeric composites in frog Rana ridibunda. Comp Biochem Physiol 2015; 172-173: 45–56.

20. Dingle JT, Barrett AJ, Weston PD. Cathepsin D. Characteristics of immunoinhibition and the confirmation of a role in cartilage breakdown. Biochem J 1971; 123: 1–13.

21. Yu C, Huang X, Xu Y, Li H, Su J, Zhong J et al. Lysosome dysfunction enhances oxidative stress-induced apoptosis through ubiquitinated protein accumulation in Hela cells. Anat Rec (Hoboken) 2013; 296: 31–39.

22. Stoka V, Turk B, Schendel SL, Kim TH, Cirman T, Snipas SJ et al. Lysosomal protease pathways to apoptosis. *J Biol Chem* 2001*;* 276*:* 3149–3157.

23. Herr I, Debatin K-M. Cellular stress response and apoptosis in cancer therapy. Blood 2001; 98: 9.

24. Faraglia B, Bonsignore A, Scaldaferri F, Boninsegna A, Cittadini A, Mancuso C,' Sgambato A. Caspase-3 inhibits the growth of breast cancer cells independent of protease activity. J Cell Physiol 2005; 202: 478–482.

25. Devarajan E, Sahin AA, Chen JS, Krishnamurthy RR, Aggarwal N, Brun AM et al. Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. Oncogene 2002; 21: 8843–8851.

26. Ruiz-Espejo F, Cabezas-Herrera J, Illana J, Campoy FJ, Vidal CJ. Cholinesterase activity and acetylcholinesterase glycosylation are altered inhuman breast cancer. Breast Cancer Res Treat 2002; 72: 11–22.

27. Zakut H, Ehrlich G, Ayalon A, Prody CA, Malinger G, Seidman S et al. Acetylcholinesterase and butyrylcholinesterase genes coamplify in primary ovarian carcinomas. J Clin Invest 1990; 86: 900–908.

28. Fuhrman B, Partoush A, Aviram M. Acetylcholine esterase protects LDL against oxidation. Biochem Biophys Res Commun 2004; 322:974–978.

29. Bay B-H, Jin R, Huang J, Tan P-H. Metallothionein as a prognostic biomarker in breast cancer. *Exp Biol Med* 2006; 231: 1516–1521.

30. Takeda A, Hisada H, Okada S, Mata JE, Ebadi M, Iversen PL. Tumor cell growth is inhibited by suppressing metallothionein-I synthesis. Cancer Lett 1997; 116**:** 145–149.

31. Tekur S, Ho S-M. Ribozyme-mediated down-regulation of human metallothionein II(a) induces apoptosis in human prostate and ovarian cancer cell lines. Mol Carcinog 2002; 33: 44–55.

32. Aiuchi T, Mihara S, Nakaya M, Masuda Y, Nakajo S, Nakaya K. Zinc ions prevent processing of caspase-3 during apoptosis induced by geranylgeraniol in HL-60 cells. *J Biochem* 1998; 124*:* 300–303.

33. Chimienti F, Seve M, Richard S, Mathieu J, Favier A. Role of cellular zinc in programmed cell death: temporal relationship between zinc depletion, activation of caspases, and cleavage of Sp family transcription factors. Biochem Pharmacol 2001; 62: 51–62.

Received: 2015-10-22

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 19–22

DOI 10.11603/ijmmr.2413-6077.2015.2.6374

QUALITATIVE COMPOSITION AND ORGANIC ACIDS CONTENT IN THE ABOVEGROUND PART OF PLANTS FROM FAMILIES *LAMIACEAE*, *ASTERACEAE*, *APIACEAE* AND *CHENOPODIACEAE*

S. M. Marchyshyn, M. I. Shanayda, I. Z. Kernychna, O. L. Demydiak, I. S. Dahym, T. S. Berdey, I. M. Potishnyj
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

Background. *Organic acids are the compounds of aliphatic or aromatic orders, which are widespread in flora and have a wide range of biological activity. We studied the qualitative composition and quantitative contents of organic acids in the aboveground part of some unofficial medicinal plants from families Lamiaceae, Asteraceae, Apiaceae and Chenopodiaceae is relevant.*

Objective. *The objects of the research are the aboveground part of unofficial medicinal plants from families Lamiaceae, Asteraceae, Apiaceae and Chenopodiaceae.*

Methods. *Identification of organic acids was performed by means of thin-layer and paper chromatography, their content was determined by means of gas chromatography, the quantitative amount of organic acids was defined by titrimetric analysis.*

Results. *In the studied raw plants the quality of organic acids and their total contents were determined (in terms of malic acid). It is established that the maximum content of organic acids is accumulated in the grass Hyssopus officinalis L. (Lamiaceae), and the minimal is in the leaves of Chrysánthemum xhortorum L. variety Apro (Asteraceae). In all studied raw plants the dominance of aliphatic acids (citric, malic, oxalic and malonic) was determined by means of gas chromatography. Benzoic is predominant among the aromatic acids.*

Conclusions. *In the studied raw plants the quality of organic acids and their total content were determined. The following results can be used in developing the methods of quality control of the studied raw plants and during the study of new bioactive substances.*

KEY WORDS: **organic acids, *Lamiaceae, Asteraceae, Apiaceae, Chenopodiaceae,* grass, leaves, thin-layer chromatography, gas chromatography, paper chromatography.**

Introduction

Organic acids are the biologically active substances which are in plants in the free state, in the form of salts, esters, dimers and compounds with other substances. They are intermediate products of plants' metabolism: involved in the oxidation of carbohydrates, fats, amino acids and proteins; used in the synthesis of amino acids, alkaloids, steroids, etc. [1, 2].

Organic acids have a wide range of biological effects. They enhance the secretory and motor activity of the digestive tract, improving digestion; help to reduce nitration processes in the organism and to reduce chemical carcinogenesis; raise the protective strength and vitality of the organism. The antioxidant, antiallergic, anti-inflammatory, antiseptic properties of these compounds are established [2].

Corresponding author: Svitlana Marchyshyn, Department of Pharmacognosy and Medical Botany, I, Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel.: +3800352520518
E-mail: marchyshyn@tdmu.edu.ua

According to the information above, we consider that the study of the qualitative composition and quantitative contents of organic acids in the aboveground part of some unofficial medicinal plants from families *Lamiaceae*, *Asteraceae*, *Apiaceae* and *Chenopodiaceae* is relevant and is of significant scientific and practical interest [3–5]. This will make it possible to justify the use of these plants in the future pharmaceutical researches.

The aim of our research is to define the qualitative composition and quantitative contents of free organic acids in the aboveground part of medicinal plants from families *Lamiaceae (Hyssopus officinalis* L., *Lophanthus anisatus* L.*)*, *Asteraceae (Bellis perennis* L. — cultivated, *Tagetes tenuifolia* Cav., *Chrysánthemum xhortorum* L. variety *Apro), Apiaceae (Angelica sylvestris* L.*)* and *Chenopodiaceae (Chenopodium album* L.*)*. The leaves of *Chrysánthemum xhortorum* and *Angelica sylvestris* and the grass of the rest species were used for phytochemical analysis. Raw plants for research were harvested during their mass flowering.

Materials and Methods

Identification of free organic acids in raw materials was performed out by means of thin-layer chromatography (TLC), paper chromatography (PC) and gas chromatography (GC) according to [6, 7]. For TLC and PC the aqueous extracts of raw plants were prepared. As standard samples for PC and GC benzoic, oxalic, malic, tartaric, succinic, salicylic, citric acids and the following solvent systems: n-butanol-formic acid-water (10:1:4), 95% ethanol-chloroform-concentrated solution of ammonia-purified water (70:40:20:2); 95% ethanol-concentrated solution of ammonia (16:4.5) and ethyl acetate-formic acid-water (3:1:1) were used. The chromatograms were developed after drying in 0.05% alcohol solution bromothymol blue and 0.1% solution of 2,6-dichlorophenolindophenol sodium salt hydrate. The action of ammonia vapours on chromatograms after a few seconds improved the contrast of spots.

The quantitative contents of organic acids in aqueous extracts of raw plants were determined according to [7] by titrimetric method. The contents of free organic acids (X) in terms of malic acid in absolutely dry raw materials in percentage were calculated by the formula:

$$X = \frac{V \times 0{,}0067 \times 250 \times 100 \times 100}{m \times 10 \times (100 - W)},$$

where: V — volume of 0.1 M sodium hydroxide solution consumed on titration, ml;

0.0067 — the amount of malic acid corresponding to 1 ml of 0.1 M sodium hydroxide solution;

m — mass of raw material, g;

W — loss in weight because of drying, %.

The quantitative contents of individual organic acids were defined by modified methods for determining the fatty acids in raw plants with further detection of organic acids. It is based on getting acid methyl esters (fatty, organic, phenolic) by methylating agent and their removal for further chromatographing by means of the gas chromatograph Agilent Technologies 6890 N with mass spectrometric detector 5973 N. Methyl esters of organic acids were obtained by a modified method of A. Carrapiso [8].

Results

3–5 organic acids were identified in the raw plants of every studied species by TLC and PC methods (Table 1).

According to Table 1, all studied species contain citric, oxalic and malic organic acids; tartaric, salicylic, benzoic and succinic acids were found only in some representatives.

The results of quantitative contents of organic acids determination (in terms of malic acid) are shown in Table 2. According to Table 2, the maximum contents of organic acids accumulate in the grass *Hyssopus officinalis*, the lowest is in the leaves of *Chrysánthemum xhortorum*.

The component composition and quantitative contents of individual organic acids in the aboveground organs of some studied species was defined by means of GS method (Table 3). 13 organic acids in raw plants of *Hyssopus officinalis*, 19 — in *Lophanthus anisatus, 5* — in *Bellis perennis, 5* — in *Chrysánthemum xhortorum, 18* — in *Chenopodium album*, 15 — in *Angelica sylvestris were determined*.

Discussion

According to the results, aliphatic organic acids (citric, oxalic, malonic and malic) were defined in all studied raw plants by means of the methods of thin-layer, gas and paper chroma-

Table 1. Results of Organic Acids Identification in Raw Plants from Families *Lamiaceae, Asteraceae, Apiaceae* and *Chenopodiaceae*

Acid	Types of plants						
	Hyssopus officinalis (grass)	Lophanthus anisatus (grass)	Bellis perennis (grass)	Tagetes tenuifolia (grass)	Chrysánthemum xhortorum (leaves)	Chenopodium album (grass)	Angelica sylvestris (leaves)
succinic	+	+	–	+	–	+	traces
tartaric	–	–	traces	–	–	–	–
citric	+	+	+	+	+	+	+
salicylic	–	traces	+	–	–	–	traces
oxalic	+	+	+	+	+	+	+
malic	+	+	traces	+	+	+	+
benzoic	+	+	–	–	–	+	–

Note: "+" — labels identified compounds, "–" — labels unidentified compounds.

Table 2. Quantitative Contents of Organic Acids in Raw Plants from Families *Lamiaceae, Asteraceae, Apiaceae* and *Chenopodiaceae*

Types of plants	Hyssopus officinalis (grass)	Lophanthus anisatus (grass)	Bellis perennis (grass)	Tagetes tenuifolia (grass)	Chrysánthemum xhortorum (leaves)	Chenopodium album (grass)	Angelica sylvestris (leaves)
Contents of acids, %	3,26±0,03	2,49±0,02	0,69±0,01	2,78±0,16	0,34±0,05	2,37±0,03	0,69±0,29

Table 3. Contents of Main Organic Acids in Raw Plants from Families *Lamiaceae, Asteraceae, Apiaceae* and *Chenopodiaceae*

Acid	Component Content, mg/kg					
	Hyssopus officinalis (grass)	Lophanthus anisatus (grass)	Bellis perennis (grass)	Chrysánthemum xhortorum (leaves)	Chenopodium album (grass)	Angelica sylvestris (leaves)
oxalic	84	370	70	310	20257	0,69±0,29
malonic	3731	3578	1069	557	963	1066
succinic	82	214	–	–	727	37
benzoic	466	101	–	–	81	–
malic	946	590	–	2797	297	1101
citric	3063	1500	438	1562	792	1122

tography. The detection of organic acids is a topical issue of phytochemical researches. The study on composition and contents of these compounds in raw plants was not the urgent matter before [2, 9, 10].

Citric acid is very widespread in nature and is used in medicine as a part of drugs to improve energy metabolism; malic and oxalic acids are used in food industry [2, 9]. We consider that among all investigated objects the grass *Chenopodium album* is the most promising source of oxalic acid, and the grass *Hyssopus officinalis* — of citric and malic.

Malonic acid is the predominant organic acid in all investigated raw plants except the grass *Chenopodium album*. This dicarboxylic acid is an important component of biochemical reactions in a plant organism and a precursor in the synthesis of cinnamic acids and flavonoids; its significant contents indicates the level of metabolism in plants during the preparation of raw plants (in the period of their flowering). Its accumulation in plants depends on the intensity of photosynthetic activity, enzymic reactions, temperature, etc. Studying the role of this acid in plants and its effect on the biological activity of phytosubstances of plants is a promising area for scientific investigations [11].

Benzoic acid is quantitatively dominant among the aromatic acids in the grass family *Lamiaceae* (Table 3), which is rather useful in pharmacy because it has anti-inflammatory, antibacterial and immunotropic properties [2]. the grass *Hyssopus officinalis* is the most promising for benzoic acid among the studied species.

Conclusions

The quantitative content of organic acids was studies and determined in the aboveground parts (grass or leaves) of the plants from families *Lamiaceae*, *Asteraceae*, *Apiaceae* and *Chenopodiaceae*.

The component composition of free organic acids in raw plants from families *Lamiaceae* (*Hyssopus officinalis*, *Lophanthus anisatus*), *Asteraceae (Bellis perennis* — cultivated, *Tagetes tenuifolia*, *Chrysánthemum xhortorum* variety *Apro)*, *Apiaceae (Angelica sylvestris)* and *Chenopodiaceae (Chenopodium album*) were analysed by means of gas chromatography for the first time. The dominance of aliphatic acids (citric, malic, oxalic and malonic) was determined. The following results can be used to develop the methods of quality control of the studied raw plants and during the study of new bioactive substances.

References

1. Dibner J, Butin P. Use of organic acids as a model to study the impact of gut microflora on nutrition and metabolism. J. Appl. Poultry Research 2002; 11 (№ 4): 453–463.

2. Kurkin VA. Pharmacognosy. Handbook for students of pharmaceutical specialties. – Samara, 2004: 202-214. (in Russian)

3. Venkateshappa SM, Sreenath KP. Potential medicinal plants of *Lamiaceae*. American Int. J. of Research in Formal, Applied & Natural Sciences 2013; 3(1): 82-87.

4. Kokanova-Nedialkova Z, Nedialkov P, Nikolov S. The genus C*henopodium*: phytochemistry, ethnopharmacology and pharmacology. Pharmacognosy Review 2009; (3): 280-306.

5. Ramya R, Mahna S, Bhamunathi S, Bhat S. Analysis of phytochemical composition and bacteriostatic activity of *Tagetes* sp. Int. Research J. of Pharmacy 2012; 3(11): 114-115.

6. Benzel' IL, Darmohrai RL, Benzel' LV. Investigation of content of ascorbic acid and free organic acids in herbal substances of Bergenia crassifolia. Pharm. journal 2010; 1: 98–101. (in Ukrainian)

7. Emelyanova IV, Kovaliov VS, Kovalev SV, Zuravel IO. Investigation of qualitative composition and dynamics of the accumulation of free organic acids in vegetative and generative organs of Grindelia squarrosa. Pharm. journal 2009; 1: 80–84. (in Ukrainian)

8. Carrapiso A, García C. Development in lipid analysis: some new extraction techniques and *in situ* transesterification. Lipids 2000; 35 (11): 1167–1177.

9. Brul S, Coote P. Preservative agents in foods, mode of action and microbial resistance mechnismes. Intl. J. Food Microbiology 1999; 50 (1–2): 1–17.

10. Chirikova NK., Olennikov DN., Rokhin AV. Organic acids from medicinal plants: *Scutellaria baicalensis (Lamiaceae).* Chem. of Nat. Compounds 2008; 44 (1): 84-86.

11. Kenneth EK. Kurt WF., Schatz FP. A one-step synthesis of cinnamic acids using malonic acid. Journal of Chem. Educ. 1990; 67 (12): 304-308.

Received: 2015-11-12

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 23–27

DOI 10.11603/ijmmr.2413-6077.2016.1.6375

SUPPLY OF ANTIHYPERTENSIVE DRUGS AND CARDIOVASCULAR MORTALITY IN POLAND IN 2000–2010

K. Barański, J. E. Zejda
MEDICAL UNIVERSITY OF SILESIA, KATOWICE, POLAND
MEDICAL SCHOOL IN KATOWICE, KATOWICE, POLAND

Background and objective. *In Poland, the sale of antihypertensive drugs has significantly increased since 2000. According to that fact, the aim of our study was to determine if the increased use of antihypertensive drugs correlates with the decreasing mortality due to cardiovascular diseases (CVD) including hypertension (HT).*

Methods. *The analysis is based on data on annual national sales (million units) of four types of antihypertensive drugs in 2000–2010. For the same period standardized mortality rates were calculated based on the data available from the Central Statistical Office in Poland. Data analysis involved correlation analysis between annual mortality rates due CVD and HT and country-wide annual sales of antihypertensive drugs (2000–2010).*

Results. *In the period 2000–2010, standardized mortality rates of CVD in the whole population followed a decreasing trend. Analysis of correlation of CVD with specific drug provided the following findings: diuretics (r=-0.97; p<0.0001) beta-blockers (r=-1.0; p<0.0001) renin-angiotensin system (RAS) inhibitors (r=-0.72 p=0.01) calcium-channel blockers (r=-0.82; p=0.001) Standardized mortality rates for the HT showed fluctuating trend. Correlations of that mortality with global sale of these drugs were no longer negative: r=0.54; p=0.08, r=0.56; p=0.08 r=0.55; p=0.07; r=0.63; p=0.03, respectively.*

Conclusions. *In Poland, in 2000–2010, an improved access to pharmacological control of HT was associated with an apparent reduction in mortality from CVD but not from HT. The latter findings might reflect imprecise definition of HT as a cause of death or the fact that HT leads to other cardiologic events usually reported as a cause of death.*

KEY WORDS: **antihypertensive drug therapy, cardiovascular disease, hypertension.**

Introduction

Standardized mortality rates caused by cardiovascular diseases (CVD) have been decreasing in developed countries since 1980 [1]. In Poland, in the last 20 years, CVD mortality decreased twice and the dynamics was strong between 1990 and 2000 [2]. Many hypotheses have addressed the issue, including changes of lifestyle and diet, improved access to modern medical technologies [3]. Another potential factor could be related to better pharmacological control of hypertension (HT) [4]. Such an explanation can be explored in ecological manner through analysis of correlation between the nation-wide sale of hypertensive drugs and CVD/HT mortality, over a period of time.

The major classes of antihypertensive drugs include beta-blockers (BB), renin-angiotensin system inhibitors (RES), diuretics (D) and calcium channel blockers (CCB). Clinical studies confirm that those drugs used in the treatment of HT are effective in preventing heart attack and stroke [5]. Less is known about population-based impact of the increased supply of the above drugs on ultimate outcome of HT, such as CVD mortality. In Poland, increased sales of and access to modern antihypertensive drugs has occurred over the two last decades and annual whole-country sales are available for a recent period (2000–2010), thus making such analysis possible.

The principal aim of our study was to determine if the increased use of antihypertensive drugs correlates with the decreasing mortality due to cardiovascular diseases (CVD) including hypertension (HT), in general population of Poland. The secondary aim of the study was to explore the hypothesized correlation in three age groups: 0–44 years, 45–64 years, above 64 years.

Materials and Methods

The analysis is based on data on annual sales (million units) of four types of antihypertensive drugs in 2000–2010: beta-blockers (BB), renin-angiotensin system inhibitors (RES),

Address for correspondence: Kamil Barański, Department of Epidemiology, Medical School in Katowice, Medyków 18th Street, Katowice, 40-752
Tel.: +48 32 208 85 38
E-mail: kbaranski@sum.edu.pl

diuretics (D) and calcium channel blockers (CCB). Annual standardized mortality rates due to CVD and HT were calculated from raw data provided by the reports of the Central Statistical Office in Poland, using direct method (reference population: WHO European Standard Population [6]. Correlation analysis of CVD and HR with drug sales (Spearman method) was performed for the entire population and for three age groups (0 to 44, 45 to 64 and over 64 years of age). Statistical significance was assumed at p=0.05. All calculations were performed in SAS software (version 9.20, SAS Institute, Cary, N.C.).

Results

Standardized mortality rates due to CVD (n/100 000) in Polish population between 2000-2010 were: 439, 428, 412, 412, 391, 377, 365, 358, 350, 350, 331, respectively. In the same period the annual sales of all antihypertensive drugs (mln units globally) were: 100, 97, 96, 100, 105, 117, 123, 134, 143, 154, 158. Figure 1 shows annual antihypertensive drugs supply in each defined category and annual mortality rates due to CVD, over the same period.

Correlation analysis of CVD mortality with the defined classes of drugs showed negative and statistically significant coefficients: BB (r=-1.0 p<0.001), RES (r=-0.72 p=0.01), D (r=-0.97; p<0.001), CCB (r=-0.82 p=0.001). Additional analyses involving stratification for age showed larger coefficients of correlation in older segments of the population (Table 1).

Standardized mortality rates due to HT (n/100 000) in Polish population between 2000–2010 were: 11,73; 10,82; 10,44; 11,30; 11,36; 12,53; 13,02; 12,60; 11,53; 12,38; 12,0; 10,24, respectively. Figure 1 shows annual antihypertensive drugs supply in each defined category and annual mortality rates due to HT, over the same period.

Trends of antihypertensive drugs supply with standardized mortality rate caused with HT are shown in Figure 2.

Table 1. Correlation of the supply of selected antihypertensive drugs (BB, RES, D, CCB) with CVD standardized mortality rate in polish population between 2000–2010

	Type of antihypertensive drug			
Specified group age	BB	RES	D	CCB
0–44 years	r=-0.82 p<0.05	r=-0.46 NS*	r=-0.756 p<0.05	r=-0.52 NS*
45–64 years	r=-0.99 p<0.05	r=-0.71 p<0.05	r=-0.96 p<0.05	r=-0.82 p<0.05
>64 years	r=-0.98 p<0.05	r=-0.72 p<0.05	r=-0.96 p<0.05	r=-0.82 p<0.05

Legends: NS – not significant, BB – beta-blockers, RES – renin angiotensin system inhibitors, D – diuretics, CCB – calcium channel blockers.

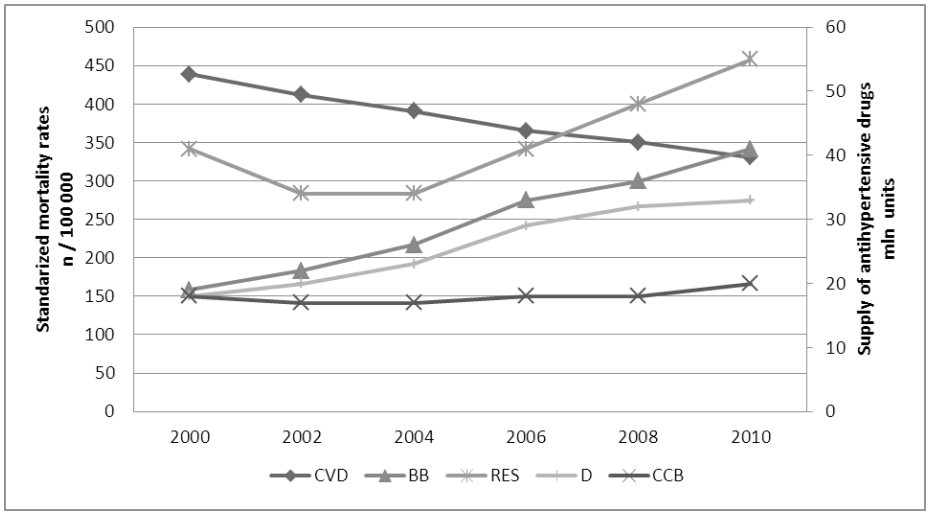

Fig. 1 Annual sales of antihypertensive drugs and CVD standardized mortality rate in 2000-2010 in polish population. Legends: CVD – cardiovascular diseases, BB – beta-blockers, RES – renin angiotensin system inhibitors, D – diuretics, CCB – calcium channel blockers.

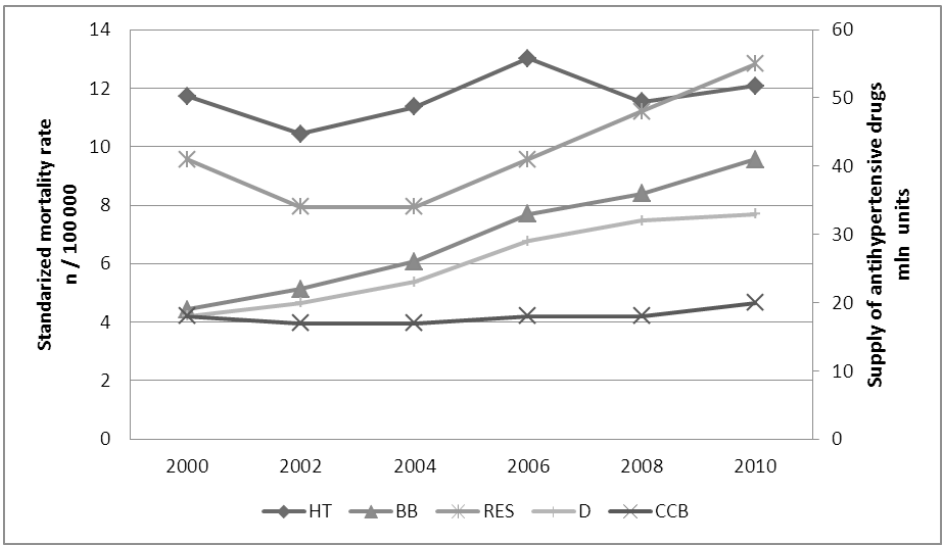

Fig. 2 Annual sales of antihypertensive drugs and HT standardized mortality rate in 2000-2010 in polish population. Legends: HT – hypertension, BB – beta-blockers, RES – renin angiotensin system inhibitors, D – diuretics, CCB – calcium channel blockers.

Correlation analysis of HT mortality with the defined classes of drugs showed negative and statistically significant coefficients: Beta-Blockers (r=0.56 p=0.07), RES (r=0.55 p=0.07), Diuretics (r=0.54; p=0.08), CCB (r= 0.63 p=0.03). Additional analyses involving stratification for age showed larger coefficients of correlation in older segments of the population (Table 2).

Discussion

High mortality due to cardiovascular diseases, seen for decades in the XX century in Poland, declined rapidly in 1991–1994. Since then the pace of decline is slower, but apparent. For example, CVD standardized mortality rates significantly decreased from 431 in 2000 to 339 in 2010. The observed phenomenon is attributed to a number of factors, including change of lifestyle with particular focus on healthy diet. Some estimates suggest that the reduction of dietary cholesterol intake in the analyzed period has explained a 39% reduction in general mortality due to coronary heart disease (CHD), in Poland [7]. Other factors include reduced smoking and increased physical activity with the estimated contribution to decreased CHD mortality at the levels of 11% and 10%, respectively [8]. Other studies suggest an important role of tobacco smoking cessation (as most important cause), followed by blood pressure control (10%) and cholesterol (10%) [9]. The role of smoking habit together with high density lipoprotein cholesterol concentrations, triglyceride concentrations, diabetes, body mass index, height, alcohol intake, physical activity, and level of education have been also confirmed by ecological findings in the Polish population [10].

Interest in the impact of environmental factors on CVD mortality is justified by given socioeconomic changes in Poland, in recent decades. However, the role of clinical control of CVD cannot be neglected. After 1990 the Polish population has an increasing access to modern therapeutical measures, including effective medical technologies and effective

Table 2. Correlation of the supply of selected antihypertensive drugs (BB, RES, D, CCB) with HT standardized mortality rate in polish population between 2000–2010

	Type of antihypertensive drug			
Specified group age	BB	RES	D	CCB
0–44 years	r=-0.58 NS*	r=-0.29 NS*	r=-0.52 NS*	r=-0.31 NS*
45–64 years	r=0.79 p<0.05	r=0.70 p<0.05	r=0.78 p<0.05	r=0.75 p<0.05
>64 years	r=0.92 p<0.05	r=0.76 p<0.05	r=0.87 p<0.05	r=0.83 p<0.05

Legends: NS – not significant, BB – beta-blockers, RES – renin angiotensin system inhibitors, D – diuretics, CCB – calcium channel blockers.

medicaments used in the treatment of CVD. In Poland, the contribution of those factors to the diminished CHD mortality could be as large as 37% [8]. The effect is likely to reflect not only a better access to modern pharmacological managements but also could be attributed to some changes in health care organization including physician-patient interactions. Social aspects could play a role and result in improved compliance of patients thus leading to stronger preventive outcomes in patients with chronic CVD [11].

Our findings showed a negative correlation between country-wide sale of antihypertensive drugs and CHD mortality, particularly in older segment of the Polish population. The result corresponds with a view concerning beneficial effect of pharmacological prevention of CHD mortality. Such effect was also seen in other study that addressed a risk of so called composite CVD events, stroke and all-cause mortality [12]. A large meta-analysis covering more than 40000 patients with hypertension provided interesting evidence concerning cardiovascular events and mortality: antihypertensive therapy was associated with the reduction of all-cause mortality rate by 13%, the risk of death from all-cardiac causes by 18%, CV events by 21, and stroke by 30%, including fatal stroke by 39% [13]. With regard to our findings it is of relevance that the gain is much larger in older patients than in young patients with hypertension [14]. It remains unknown to what extent the age-related difference reflects age-related duration of disease and, consequently, age-related duration of the treatment. Such explanation cannot be ignored when discussing no apparent effect of the treatment of hypertension on CHD mortality over the period of 4-5 years [15].

Our findings did not show major differences between correlations provided by different groups of antihypertensive drugs analyzed in the study. The study protocol hampers a more detailed discussion of that point. The ecological approach used in our analysis has well-known limitations but a general conclusion about a beneficial impact of increased access of the population to modern antihypertensive therapy on a risk of CVD mortality in that population seems to be convincing. We did not see a similar effect in relation to HT mortality. A lack of such effect could be explained by a relatively infrequent occurrence of HT as a reported cause of death in Poland. Death certificates usually include fatal complication of HT thus making this diagnosis an unreliable index of cause-specific mortality, with a very limited pertinence to conclusive statistical analyses.

Conclusions

In Poland, an improved access of the population to pharmacological control of HT, as suggested by increased country-wide sale of modern antihypertensive drugs, is associated with an apparent reduction in mortality from CVD but not from HT. The latter finding might reflect imprecise definition of HT as a cause of death or the fact that HT leads to other cardiologic events usually reported as a cause of death. The findings seem to confirm an important contribution of pharmacological measures in prevention of CVD mortality, in addition to the well explored role of socio-economic and life-style factors, in Poland.

References:

1. Danaei G, Finucane MM, Lin JK, Singh GM, Paciorek CJ, Cowan MJ, et al. National, regional, and global trends in systolic blood pressure since 1980: systematic analysis of health examination surveys and epidemiological studies with 786 country-years and 5.4 million participants. Lancet 2011; 377: 568–577.

2. Barański K, Zejda JE.: Nadciśnienie tętnicze jako główna przyczyna zgonów w Polsce w latach 1990-2010. Nadciśnienie tętnicze, 2014; 18(2): 74.

3. Cheng A, Braunstein JB, Dennison C, Nass C, Blumenthal RS. Reducing global risk for cardiovascular disease: using lifestyle changes and pharmacotherapy. Clin Cardiol. 2002; 25(5): 205–12.

4. Gudmundsson LS, Johannsson M, Thorgeirsson G, Sigfusson N, Sigvaldason H, Witteman JC. Hypertension control as predictor of mortality in treated men and women, followed for up to 30 years. Cardiovasc Drugs Ther. 2005; 19(3): 227–35.

5. White WB.: Update on the drug treatment of hypertension in patients with cardiovascular disease. Am J Med. 2005; 118(7): 695–705.

6. WHO age standardization of rates: a new who standard: Ahmad OB, Boschi-Pinto C, Lopez AD, Murray C, Lozano R, Inoue M.: GPE Discussion Paper Series: No.31 EIP/GPE/EBD World Health Organization 2001.

7. Zatonski WA, Willett W.: Changes in dietary fat and declining coronary heart disease in Poland: population based study BMJ 2005; 331:187.

8. Bandosz P, O'Flaherty M, Drygas W, Koziarek J, Wyrzykowski B, Rutkowski M, et al.: Explaining the decline in coronary heart disease mortality in Poland between 1991 and 2005 ESC Congress, Stockholm, Sweden (2010 28 Aug–01 Sep) Abstract 1119.

9. Unal B, Critchley JA, Capewell S.: Explaining the decline in coronary heart disease mortality in England and Wales between 1981 and 2000. Circulation, 109 (2004): 1101–1107.

10. Zatonski WA, McMichael AJ, Powles JW, Ecological study of reasons for sharp decline in mortality from ischaemic heart disease in Poland since 1991 BMJ. 1998 Apr 4; 316(7137): 1047–105.

11. Papp R, Csaszar A, Paulik E, Balogh S.: Correlations between prescription of anti-hypertensive medication and mortality due to stroke. BMC Cardiovascular Disorders 2012, 12: 1.

12. Thompson AM, Hu T, Eshelbrenner CL, Reynolds K, He J, Bazzano LA.: Antihypertensive treatment and secondary prevention of cardiovascular disease events among persons without hypertension: a meta-analysis. JAMA. 2011; 305(9): 913–22.

13. Ostrowski M, Zanchetti A, Nikfar S, Muntner P, Aronow WS, Howard VJ, Wong ND, Howard G, Abdollahi M, Banach M.: The effect of hypertension pharmacotherapy in older adults. The results of a meta-analysis of 11 randomized control trials with 40325 patients. European Heart Journal (2014) 35 (Abstract Supplement), 1192.

14. Perez MI, Musini VM, Wright JM.: Effect of early treatment with anti-hypertensive drugs on short and long-term mortality in patients with an acute cardiovascular event. Cochrane Database Syst Rev. 2009 Oct 7;(4):CD006743.

15. Diao D, Wright JM, Cundiff DK, Gueyffier F. Pharmacotherapy for mild hypertension. Cochrane Database of Systematic Reviews 2012, Issue 8.

Received: 2015-03-11

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 28–31

DOI 10.11603/ijmmr.2413-6077.2016.1.6376

EPIDEMIOLOGICAL AND EPIZOOTIC ASPECT OF LEPTOSPIROSIS EVOLUTION IN TERNOPIL REGION

N. A. Vasylieva, Yu. A. Kravchuk
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE
TERNOPIL REGIONAL LABORATORY CENTRE OF THE STATE SANITATION AND EPIDEMIOLOGICAL SERVICE OF UKRAINE, TERNOPIL, UKRAINE

Background. *Ternopil region is endemic on leptospirosis. Its natural conditions (slightly alkaline or alkaline soils, air temperature, sufficient rainfall) contribute to the existence of major natural reservoir of the pathogen – mouse-like rodents. In the region, different serovariants of leptospira are exuded by rodents and farm animals.*

Objective. *The materials of the Department of Highly Infectious Diseases of Ternopil Regional Laboratory Centre of the State Sanitation and Epidemiological Service of Ukraine, Ternopil Regional Laboratory of Veterinary Medicine, Clinic of Infectious Diseases of TSMU were studied.*

Leptospiras were detected by dark ground microscopy (DFM) of blood of patients, trapped rodents and examined farm animals.

Results. *The circulating of pathogens between different sources (rodents, animals) and annual disease incidence evidences that new leptospira serovar are carried onto endemic area mostly by farm animals; humans are infected from them through the environment sometimes in 3-5 years intervals; the further diffusion to the new areas of this pathogen serovars in all kinds of the examined mouse-like rodents is noticed.*

It is established that farm animals and rodents are competing reservoirs. To predict the future epidemiological situation of leptospirosis among the humans and to improve its diagnosis the constant monitoring of the population, infection and leptospira carriage among mouse-like rodent and farm animals and expanding of the panel of diagnostic leptospira strains including new pathogen variants in animals is necessary.

Conclusions. *The development of additional reservoirs in animals, with circulating of other pathogen serovars among them, such as mouse-like rodents, which were previously absent in the main natural reservoir, cause the change of etiological structure in human leptospirosis at the endemic areas. The range of human leptospirosis pathogens and its further spreading among all kinds of rodents increased during our research. The results of detection of leptospirosis pathogens among the various contingents which were studied evidence that the farm animals and rodents are competing reservoirs that cause human infection through environment.*

KEY WORDS: **leptospirosis, disease incidence, source of infection, rodents, farm animals.**

Introduction

Leptospirosis is a common infectious disease among humans and animals. The features of this disease are: mostly severe course, high mortality, and great social and economic losses. Every year more than 1.03 million cases and 58.900 deaths happen due to leptospirosis in the world. The highest incidence and mortality rate is in South and Southeast Asia, Oceania, Latin America, East Africa [1]. This disease was recorded in almost all regions of Ukraine, in Ternopil region as well.

Corresponding author: Nataliya Vasylieva, Department of Infectious Diseases and Epidemiology, Dermatology and Venereology, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel.: +380352524725
E-mail: vasylyeva@tdmu.edu.ua

The mouse-like rodents and natural conditions (slightly alkaline and alkaline soils, sufficient rainfall, appropriate air temperature [2, 3]) as main natural pathogen reservoir define the endemicity of leptosirosis area and contribute to preservation and dissemination of this disease.

The **aim** of the research was to investigate regularities of epidemic and epizootic processes of leptospirosis and study it's pathogen circulation between different nidi (rodents, animals) and person.

Materials and Methods

The materials of the Department of Highly Infectious Diseases of Ternopil Regional Laboratory Centre of the State Sanitation and Epidemiological Service of Ukraine, Ternopil Re-

gional Laboratory of Veterinary Medicine, Clinic of Infectious Diseases of TSMU were studied.

Leptospiras were detected by dark ground microscopy (DFM) of blood of patients, trapped rodents and examined farm animals.

Pathogen serovar was determined according to serological investigation in microhemagglutination test of live leptospira cultures (RMAL); standard diagnostic set consisted of 11 leptospira serogroups that consisted of both diagnostic strains proposed by the WHO and strains registered in Ukraine and identified in accordance with international reference cultures.

The leptospirosis incidence among the humans in Ternopil region in 1980-2014, leptospira contamination of animals at private and collective farms and also rodents in the wild were studied.

Results and Discussion

In Ternopil region there were two rather large "bath" leptospirosis outbreaks in children (laboratory deciphered L. grippotyphosa) in July 1963 (18 patients) and in August 1972 (22 patients). It happened after swimming in the river, livestock farms were situated upstream of it. The rodents trapped near the river contaminated with leptospira too.

Since 1972 regular monitoring of leptospirosis among the humans took place. The leptospirosis incidence among humans in the region differed: 1,05–12,17 per 100 thousand of population (2–149 cases per year); the highest rate was in 1992–2001, the maximum incidence was in 1994, in 2014 – 3,26, which exceeded the average rate in the country all the time and was the highest in Ukraine for many years.

Till 1981 L. grippotyphosa was the main leptospirosis pathogen in humans (90% cases etiologically deciphered). The percentage of this leptospira in the structure of leptospirosis decreased by 41,1–51,4% in 1988-1989, by 3.4% in 1994. From 1999–2000 there were no incidence of leptospirosis caused by this pathogen. L. icterohaemorrhagiae was the main etiological factor of this disease in the 90s (in 1991 – 93,2%, in 2000 – 100,0%).

The disease was rarely caused by other leptospira serogroups. The disease caused by L. hebdomadis (5,9–45,4%), L. canicola (14,2–60,0%), L. pomona (3,1–21,4%) was registered in 2002; the rate of L. icterohaemorrhagiae decreased by 37.5%, there were only some rare cases of L. grippotyphosa (Fig. 1). The rate of combined leptospirosis significantly increased in recent years (34.3% of all registered in 2014).

The etiologic spectrum of leptospirosis in humans and in mouse-like rodents in the first stage of observation did not coincide (1981–1993). In the natural habitats the antibodies to leptospira of Grippotyphosa serogroup were found in mostly different kinds of voles, field and house mice; only to Icterohaemorrhagiae – in domestic grey rats. Hebdomadis segroup was also found in voles and mice and at home foci as well. During the same period the disease caused by the above mentioned pathogens as well as Canicola and Pomona (1982) (Fig. 1), further Kabura (2005) Polonica (2007), was registered in humans, but in rodents these pathogens had not been found before that

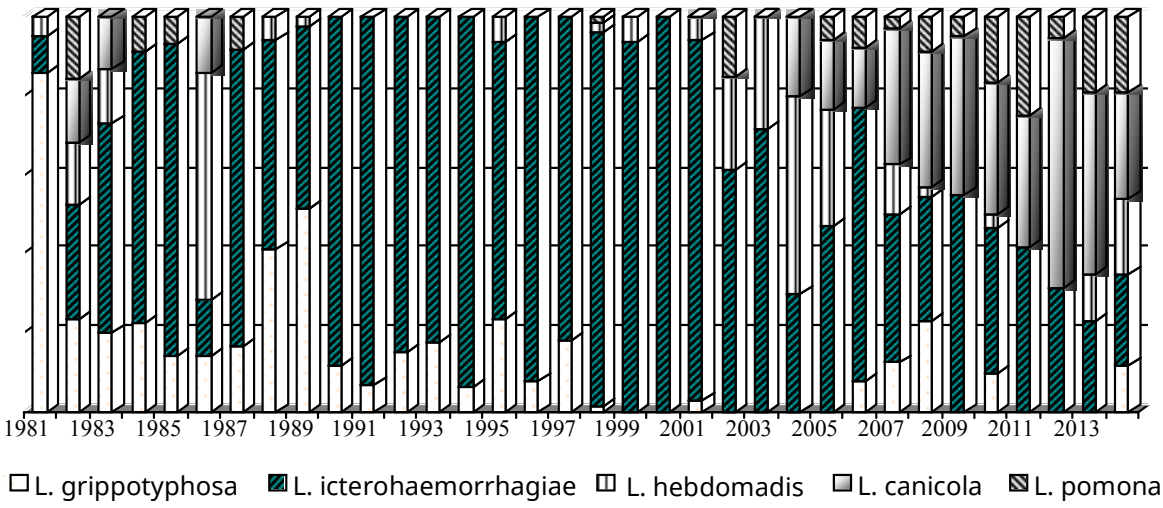

Figure 1. Etiological structure of human leptospirosis incidence in Ternopil region (annual percentage fraction of pathogens, 1980-2015, %).

time. In 2010 one case of leptospirosis in humans caused by L. australis was registered. According to the epidemiological anamnesis the source of infection has not been established. The contamination possibly happened at other area.

The animals can be the source of leptospirosis infection too. The vaccination process, natural infection or animal disease might cause their serological positivity. The vaccine do not contain L. kabura, (L. hebdomadis), L. bratislava, which are often detected in animals.

Every year the carriers of leptospirosis and disease incidence in farm animals were registered at private and collective farms of Ternopil region which ended up with animal death and abortion; from 1983 epizootic diseases were rare, clinically apparent forms of leptospirosis were not registered, however, sufficiently high titers (1: 400) of antibodies were defined, which evidenced about carrying or subclinical form of the disease in animals and we should consider them as potential sources of leptospirosis for humans. It is possible that the animals' infection happened after restocking from other farms and areas.

According to the recent researches, the duration of leptospirosis carrying in cattle is up to 6 months: in pigs – up to1 year; 10% of cattle and 30% of pigs are carriers for life, as well as rats and mice [4]. However, some authors believe that at most areas the domestic and farm animals are not very important for the epidemiology of leptospirosis because rats usually are revealed at these loci [5].

Etiological structure of animal leptospirosis is diverse and includes L. icterohaemorrhagiae, pomona, grippotyphosa, hebdomadis, polonica, canicola, kabura, tarassovi; L. bratislava was registered in 2007 (87.5% in pigs in 2008), often diagnostically significant titers of antibodies to two different serovars are defined at the same time (71,4–78,0% in cattle in 2003–2006).

Till 1983 only leptospira of Grippotyphosa and Icterohaemorrhagiae serogroups, sometimes of Hebdomadis, was detected; later antibodies to other serovars were identified: Canicola in rats (2004), Kabura (2006) in different kinds of voles, except Grippotyphosa, also Icterohaemorrhagiae, Pomona, Canicola, Hebdomadis; the expanding of pathogens and their further distribution in all kinds of examined mouse-like rodents took place. In 2015 leptospira Polonica was identified in rodents for the first time.

The range of serologically identified pathogens was listed during the monitoring of humans, farm animals and mouse-like rodents (Table 1).

Due to this study and our previous researches [6–8] we verify that leptospira serovars in farm animals cause the human diseases because these pathogens were not exuded by rodents in this region before. New leptospira serovars, which are carried mostly by farm animals and humans are infected through environment from them, in humans are usually registered in 3–5 years after they are exuded from animals. We also evidence the further spread of the new pathogen serovars at this area in all kinds of examined mouse-like rodents.

So, it was proved that farm animals and rodents are competing reservoirs of leptospirosis pathogens. To predict future epidemiological situation of leptospirosis in humans and to improve its diagnosis the monitoring of the population, infection and leptospira carriage among mouse-like rodents and farm animals and expanding of the panel of diagnostic leptospira strains including new pathogen variants in animals is necessary.

Table 1. Leptospirosis pathogens serologically identified in humans, farm animals and rodents (1972-2015).

Rodents	Farm animals	Humans
L. icterohaemorrhagiae (1972)	L. icterohaemorrhagiae (1972)	L. icterohaemorrhagiae (1972)
L. grippotyphosa (1972)	L. grippotyphosa (1972)	L. grippotyphosa (1972)
L. hebdomadis (1983)	L. hebdomadis (1981)	L. hebdomadis (1981)
L. canicola (1982, 2004)	L. canicola (1979)	L. canicola (1982)
L. pomona (2008)	L. pomona (1979)	L. pomona (1982)
L. kabura (2006)	L. kabura (2002)	L. kabura (2005)
L. polonica (2015)	L. polonica (2002)	L. polonica (2007)
	L. tarassovi (2002)	
	L. bratislava (2007)	
		L. australis (2010)

Thus, the factors that contribute to the evolution of the epidemic process in leptospirosis are:

– biological changes of the natural reservoir – species composition of rodents, their number and contamination;

– expanding of new leptospira strains into endemic areas of farm animals, which cause human infection through environment and further spread among mouse-like rodents and pathogen establishment there.

Prevention: annual examination for leptospirosis of breeding stock and animals purchased for sale in other farms; sterilization of animal carriers; vaccination; diratisation; draining of wetlands could decrease (possibly avoid) the expanding of new leptospira strains into endemic areas and prevent the disease incidence in humans.

Conclusions

1. The development of additional reservoirs in animals, with circulating of other pathogen serovars among them, such as mouse-like rodents, which were previously absent in the main natural reservoir, cause the change of etiological structure in human leptospirosis at the endemic areas.

2. The range of human leptospirosis pathogens and its further spreading among all kinds of rodents increased during our research.

3. The results of detection of leptospirosis pathogens among the various contingents which were studied evidence that the farm animals and rodents are competing reservoirs that cause human infection through environment.

References

1. Costa F, Hagan JE, Calcagno J et al. Global Morbidity and Mortality of Leptospirosis: A Systematic Review. PLoS Negl Trop Dis 2015; 9 (9): 3898.

2. Виноград НО, Кіріяк ОП, Мурзова ЛІ та ін. Еколого-епідеміологічні особливості лептоспірозу на Івано-Франківщині. Сучасні інфекції 2004; 1: 60-65.

3. Кравчук ЮА, Васильєва НА. Епізоотолого-епідеміологічні особливості лептоспірозу в Тернопільській області. Анали Мечниківського інституту 2015; 2: 165-171.

4. Задорожна ВІ, Протас СВ, Гопко НВ та ін. Епізоотологічні та епідеміологічні аспекти лептоспірозу в Україні. К; 2014: 46.

5. Бернасовська ЄП, Кондратенко ВМ, Мельницька ОВ. Проблема лептоспірозу в Україні. Інфекційні хвороби 1996; 2: 37-39.

6. Васильєва НА, Буртняк ТВ, Блажкевич БВ, Грузина ЛО. Захворюваність людей на лептоспіроз та інфікованість патогенними лептоспірами гризунів у Тернопільській області. Інфекційні хвороби 1995; 2: 22-25.

7. Васильєва НА, Поліщук ЮА, Івахів ОЛ та ін. Епідеміологічні особливості лептоспірозу в західному регіоні України. Інфекційні хвороби 2008; 2: 14-18.

8. Васильєва НА, Луцук ОС, Павлів ОВ. Еволюція епідемічного процесу лептоспірозу (за матеріалами Тернопільської області). Профілактична медицина 2011; 2: 69-73.

Received: 2016-02-19

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 32–38

DOI 10.11603/ijmmr.2413-6077.2016.1.6379

ENZYME MARKERS ACTIVITY AND BILE FORMATION FUNCTION OF LIVER IN CASES OF TUBERCULOSTATICS AND HEXAVALENT CHROMIUM COMPOUNDS AFFECTION IN RATS

N. I. Burmas, L. S. Fira, P. H. Lyhackyy
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

Background. *Currently, the growing incidence of toxic lesions of the liver is associated with industrial chemicalization and uncontrolled use of hepatotoxic drugs in everyday life. There are about one thousand drugs with high or low hepatotoxicity, such as anti-TB drugs.*

Objective. *In this research we studied the intracellular enzymes activity and bile formation function of the liver in rats of different ages in cases of tuberculostatic (isoniazid and rifampicin) affection and chromium (potassium dichromate) intoxication.*

Methods. *The experimental affection of rats of different ages was performed by combined injection of hexavalent chromium compounds (a solution of potassium dichromate, 3 mg/kg), isoniazid (0.05 g/kg) and rifampicin (0.25 g/kg). On the 7th and 14th days the rats were injected with enterosorbent Sorbex (150 mg/kg). Enzyme markers activity of the liver was evaluated due to alanine and aspartate aminotransferases (ALT and AST) and alkaline phosphatase (ALP) rates. Bile formation function of the liver was evaluated by total bilirubin and bile acids content in blood.*

Results. *The disorders in hepatocytes plasma membranes permeability were defined by the increased rates of ALT, AST and alkaline phosphatase in blood serum which were decreased in the liver. It was determined that total bilirubin and bile acids content in blood serum of the affected animals increased. It influenced hepatocytes excretion in bile capillaries and caused cholestasis and revenues decrease in bile.*

Conclusions. *The most significant metabolic disorders in cases of chrome-isoniazid-rifampicin affection were defined in immature and senior animals in comparison with mature animals.*

KEY WORDS**: isoniazid, rifampicin, hexavalent chromium compounds, liver enzymes, bile formation.**

Introduction

Considering the role of liver in the chemical compounds metabolism we can assert that there are no drugs that in certain conditions would not cause impairment. More and more information about their hepatotoxic effect indicates that medical affection of the liver is one of the major problems of hepatology [1–3]. Antituberculosis drugs are of great interest [4–6].

According to the Centre of monitoring of adverse reactions of drugs (2007), isoniazid – 29.2%, rifampicin – 26.7%, capreomycin – 17.1%, ethambutol – 10.2% dominate among monopreparations in high incidence of the adverse reactions in world [5]. Thus, the risk of the development of hepatitis increases in patients who take rifampicin together with isoniazid. In this case hepatitis incidence is 5-8%. During the isoniazid monotherapy, the incidence of hepatitis is 1.2%, but during the rifampin monotherapy – 0.3% [4].

According to the researches [7, 8], the hepatotoxicity of isoniazid may be developed in two ways: 1. the accumulation of free radicals with activation of lipid peroxidation and the formation of reactive metabolites: acetylisoniazid, hydrazine, monoacetylhydrazine; 2. the increased activity of N-acetylisoniazid by N-hydroxylation and formation of acetyl radical and acetyl carbonium ion.

The metabolism of acetyl hydrazine and microsomal monooxygenases cause hepatotoxic effect as a result of the covalent addition of

Address for correspondence: Nataliya Burmas, Department of General Chemistry, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel.: +380976123434
E-mail: burmas@tdmu.edu.ua

acetyl groups to the liver proteins [7], which manifest as temporary asymptomatic increase of transaminases activity. The hepatotoxicity of rifampin is also due to the formation of toxic metabolites as a result of its deacetylation in the liver that leads to hepatocytes dystrophy [9, 10].

However, increasing saturation of the environment by the compounds of heavy metals leads to the accumulation of these compounds in human bodies, of those who live on the contaminated areas from early childhood [11–13]. According to some studies, in the future the compounds of heavy metals as the threat for the environment can become the urgent matter, leaving nuclear stations wastes and organic anthropogenic pollution behind [13].

Today Chromium (VI) is recognized by the International Agency of the Cancer Researches of the European Union to be one of six chemical elements (Arsenic, Beryllium, Cadmium, Cobalt, Nickel and Chromium) that reveal the carcinogenic effect on human body [14, 15]. The influence of the compounds of Chromium (VI) is accompanied by toxic effects and serious internal organs (kidneys, lungs, liver) damage.

But, the recent studies do not provide full concept of the of liver enzymes activity and its influence on bile formation function in cases of combined effect of hexavalent chromium compounds on age-dependent isoniazid-rifampicin destruction of the body, which is presented in this research.

Materials and Methods

The experiments were conducted on outbred white male rats of three age groups: the 1st group – immature (3-month-old animals, 90–110 g in weight); 2nd group – mature (6-month-old animals, 150–170 g in weight); and the 3rd group – senile (18-months-old animals, 280–300 g in weight). The rats were kept on a standard diet at the vivarium of Ternopil State Medical University.

The manipulations on the animals were carried out according to the Article 26 of the Law of Ukraine "On protection of cruelty to animals" from 21.02.2006. No. 3447-IV, "European Convention on protection of vertebrate animals used for experimental and other scientific purposes", "General ethical principles of the experiments on animals", approved 20.09.2001 during the 1st Ukrainian National Congress on Bioethics considering regulations of NIH Guide on Care and Use of Laboratory Animals [16].

The experimental toxic affection of the animals was simulated by combined effect of hexavalent chromium compounds, isoniazid and rifampicin. Hexavalent chromium compounds were administered intragastrically to animals every day for one group by the solution of potassium dichromate, 3 mg/kg. For another group on the 7th and 14th days isoniazid and rifampicin by metallic probe in aqueous solution, 0.05 g/kg and 0.25 g/kg accordingly, were administered intragastrically. For combined action, the xenobiotics mentioned above were administered in the same doses.

Euthanasia was performed by means of thiopental sodium on the 7th and 14th days from the first day of xenobiotics administration. The study of liver homogenate and blood serum was performed. The blood was taken from the heart of animals by centrifugation at 3000 rev/min during 30 min. The obtained blood serum, sedimentary liquid, was used for researches. Liver (250 mg) was put into 10% homogenate and different homogenisation methods were used after previous perfusion in physiological solution.

The activity of liver enzyme markers was determined by the rate of aminotransferases (ALT and AST) and an alkaline phosphatase (ALP) (the reagents of OOO NPP Filisit- Diagnostics, Ukraine) in blood serum and liver homogenate. The evaluation of ALT rate was conducted by compound of 2-oxoglutaric acid and L-alanine, which under the influence of alanine aminotransferase formed L-glutamic and pyruvic acids. The interaction of pyruvic acid and 2.4-dynitrophenylhydrazine in alkaline medium formed 2.4-dynitrophenylhydrazones that had high coefficient of the molar extinction, so its optical density registered on the FEC was directly proportional to the activity of the enzyme.

The enzyme activity was estimated by the calibration graph due to the content of pyruvic acid in mkmol/(L·h) [17]. AST rate was evaluated by optical density measuring of 2.4 nitrophenylhydrazones of 2-oxoglutaric and pyruvic acids in alkaline medium. Hydrazone of pyruvic acid has a higher coefficient of the molar extinction, so there is a directly proportional relationship of optical density of the reaction solution to the enzyme activity. The enzyme activity was evaluated by the calibration graph due to the content of pyruvic acid, mkmol/(L·h) [17]. Estimation of the alkaline phosphatase rate was based on the property of the enzyme to hydrolyse the etheric bond in β-glycero-

phosphate and eliminate the phosphoric acid. Phosphorus was determined by colorimetric method due to the reaction with molybdenum reagent in the presence of a reducing eikonogen or ascorbic acid. The product of reaction was molybdenum blue; its colour intensity was directly proportional to the amount of phosphorus in the simple evaluation of the enzyme activity [18].

The bile formation function of the liver in the animals was defined by the content of total bilirubin and bile acids in blood serum. The content of total bilirubin was determined by caffeine reagent, which together with diazotized sulphanilic acid formed azobilirubin of pink-purple colour. The colour intensity of this solution was directly proportional to the concentration of total bilirubin in the sample. Evaluation of total bilirubin in blood serum was performed by the calibration graph, mmol/L [18]. Determination of bile acids content was based on the reaction of colour products formation by condensation, which interacted with bile acids and oxymethyl furfural. These solutions were obtained from fructose. They are the products of hydrolysis by adding concentrated sulfuric acid to sucrose. Bile acids content was evaluated by the calibration graph due to the tauroholic acid content, g/L [18].

The statistical processing of the results was performed on a PC by means of programs "Microsoft Excel" and "STATISTICA 6.0" on the basis of arithmetic middling and errors according to Student's t-test [19]. The changes were considered to be reliable at $p \leq 0.05$.

Results

We noted the increased ALT rate (Table 1) in the blood serum of animal groups of all ages. It was the highest in rats affected by tuberculostatics and potassium dichromate. On the 14th day of xenobiotics administration the rate increased in 2.9 times in immature animals in comparison with the control group, in 2.5 times – in mature animals and in 3.2 times – in senior animals. The mature animals, which were affected by hexavalent chromium compounds, proved to be the least sensitive. Their affection rate exceeded the normal range in 2.3 times.

In the immature rats the ALT rate increased by 120% on the 7th day of the research and by 145% – on the 14th day of the affection by isoniazid and rifampicin, in the mature animals this rate exceeded the level of the intact control by 30% on the 7th day and by 128% till the end of the experiment.

The senior animals were more sensitive to anti-TB drugs. ALT rate in blood serum increased by 181% on the 14th day after the affection.

The most significant changes were observed in the liver of the senior animals which underwent the aforementioned xenobiotics

Table 1. Alanine aminotransferase rate in blood serum (mkmol/L·h) and liver (mkmol/kg·h) of rats affected by isoniazid, rifampicin and hexavalent chromium compounds, (M±m)

Research material	Group of animals	Age group of animals					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
blood serum	intact control, n=6	0.83±0.05		2.96±0.18		2.34±0.15	
	affected by potassium dichromate, n=6	1.51±0.10*	1.93±0.16*	4.03±0.32*	6.66±0.17*	3.75±0.36*	6.16±0.37*
	affected by isoniazid and rifampicin, n=6	1.83±0.11*	2.03±0.20*	3.84±0.24*	6.74±0.26*	4.44±0.32*	6.58±0.30*
	affected by potassium dichromate, isoniazid and rifampicin, n=6	2.21±0.12*	2.38±0.14*	4.69±0.19*	7.46±0.42*	5.87±0.30*	7.57±0.26*
liver	intact control, n=6	5.40±0.12		8.30±0.21		6.08±0.33	
	affected by potassium dichromate, n=6	3.96±0.26*	2.97±0.16*	6.55±0.23*	3.96±0.26*	2.97±0.16*	6.55±0.23*
	affected by isoniazid and rifampicin, n=6	3.68±0.20*	2.83±0.11*	6.48±0.27*	3.68±0.20*	2.83±0.11*	6.48±0.27*
	affected by potassium dichromate, isoniazid and rifampicin, n=6	3.18±0.22*	2.42±0.12*	5.78±0.31*	3.18±0.22*	2.42±0.12*	5.78±0.31*

Note: here and in the following tables * – significant differences between the animals of intact controls and the affected animals, $p \leq 0.05$.

compounds. ALT rate decreased till the end of the experiment by 58% (p≤0.05) in this group, when in the immature group – by 55%, in the mature one – by 45% in comparison with animals of the intact control.

In blood serum of the experimental animals, affected by $K_2Cr_2O_7$, the AST rate was gradually increasing and reached the highest rate at the end of the experiment by 251% in the immature animals, by 181% – in the mature animals and 267% – in the senior animals if compared to the control group of animals (Table 2). In the immature and mature rats AST rate increased in 3.0 and 2.1 times accordingly in comparison with the control group.

During the research the decrease in AST rate in liver of the affected animals of all age groups was determined (Table 2). In the immature animals the activity of this enzyme decreased by 17% on the 7th day of the research after the administration of $K_2Cr_2O_7$, in the mature animals – by 33% and in the senior animals – by 39%. It was proved that the accumulation of heavy metals, which got into the body of animals from the environment, took place in hepatocytes [11, 12].

We studied alkaline phosphatase rate in blood serum and liver of the affected rats (Table 3). It was established that this enzyme is a marker of liver disorder and indicates the inflammation in it.

The administration of potassium dichromate into the body of the immature animals caused the increase of alkaline phosphatase rate in blood serum in 1.3 times on the 7th day of the experiment, in the mature animals – in 1.5 times and in the senior animals – in 1.3 times, that caused toxic affection of liver. These changes were significant (p≤0.05).

We evidenced the highest rate of alkaline phosphatase in blood serum of the immature animals after combined effect of potassium dichromate, isoniazid and rifampicin, which was 100% on the 7th day after affection and 127% – on the 14th day in comparison with the animals of the intact control.

In liver of the affected animals the ALP rate decreased during the experiment in all experimental groups (Table 3). The lowest rate of alkaline phosphatase was on the 14th day of the research in the mature animals after the combined effect of xenobiotics (772.86±29.62) nmol/(s·g) that is in 1.7 times lower than in the control group (1338.23±54.21 nmol/(s·g).

We evidenced a significant increase (p≤0.05) in total bilirubin content in blood serum of the animals of all age groups in comparison with control rats (Table 4). On the 14th day of the experiment the total bilirubin content increased by 53% in the immature animals, by 40% in the mature ones and by 28% in the senior rats after the affection with anti-TB drugs in comparison with the animals of the intact control.

We evidenced the highest content of total bilirubin at the end of the research in the mature animals after combined administration of $K_2Cr_2O_7$, isoniazid and rifampicin, which was 206% in comparison with intact animals that is

Table 2. Aspartate aminotransferase rate in blood serum (mkmol/L·h) and liver (mkmol/kg·h) of rats affected by isoniazid, rifampicin and hexavalent chromium compounds, (M±m)

Research material	Group of animals	Age group of animals					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
blood serum	intact control, n=6	0.70±0.06		0.64±0.04		1.05±0.20	
	affected by potassium dichromate, n=6	0.94± 0.04*	1.76± 0.08*	0.93± 0.05*	1.16± 0.04*	2.04± 0.14*	2.80± 0.09*
	affected by isoniazid and rifampicin, n=6	1.05± 0.05*	1.84± 0.10*	1.10± 0.05*	1.21± 0.06*	2.61± 0.09*	3.40± 0.14*
	affected by potassium dichromate, isoniazid and rifampicin, n=6	1.21± 0.07*	2.07± 0.14*	1.19± 0.06*	1.34± 0.08*	3.22± 0.14*	3.85± 0.18*
liver	intact control, n=6	1.21±0.11		2.66±0.06		2.48±0.12	
	affected by potassium dichromate, n=6	1.01± 0.05	0.91± 0.02*	1.79± 0.06*	1.44± 0.05*	1.52± 0.04*	1.25± 0.04*
	affected by isoniazid and rifampicin, n=6	1.02± 0.04	0.86± 0.03*	1.66± 0.10*	1.34± 0.07*	1.45± 0.04*	1.18± 0.03*
	affected by potassium dichromate, isoniazid and rifampicin, n=6	0.87± 0.03*	0.75± 0.04*	1.45± 0.10*	1.25± 0.06*	1.34± 0.04*	1.13± 0.04*

Table 3. Alkaline phosphatase rate in blood serum (nmol/s·L) and liver (nmol/s·g) of rats affected by isoniazid, rifampicin and hexavalent chromium compounds, (M±m)

Research material	Group of animals	Age group of animals					
		immature		mature		senior	
		Research duration, days					
		7th	14th	7th	14th	7th	14th
blood serum	intact control, n=6	1924.64±113.32		2405.80±159.13		3007.25±240.58	
	affected by potassium dichromate, n=6	2766.67± 159.13*	3518.48± 227.04*	3488.41± 206.60*	3849.28± 184.40*	3939.49± 205.29*	4360.51± 225.44*
	affected by isoniazid and rifampicin, n=6	3247.83± 192.09*	3849.28± 206.60*	4210.15± 159.13*	4540.94± 142.96*	4330.43± 208.35*	4781.52± 137.81*
	affected by potassium dichromate, isoniazid and rifampicin, n=6	3849.28± 159.13*	4360.51± 182.92*	4691.30± 104.17*	4751.45± 144.85*	4961.96± 137.81*	5352.90± 159.13*
liver	intact control, n=6	739.78±30.90		1338.23±54.21		1705.11±74.21	
	affected by potassium dichromate, n=6	634.53± 20.53*	610.47± 14.30*	1004.42± 50.54*	878.12± 33.16*	1377.32± 41.84*	1305.14± 19.02*
	affected by isoniazid and rifampicin, n=6	562.35± 19.44*	496.20± 20.17*	941.27± 38.82*	836.01± 35.68*	1341.23± 30.79*	1235.14± 16.64*
	affected by potassium dichromate, isoniazid and rifampicin, n=6	514.24± 21.22*	457.10± 13.72*	878.12± 20.66*	772.86± 29.62*	1205.91± 26.93*	1106.67± 22.18*

Table 4. Total bilirubin content in blood serum (mkmol/L) of rats affected by isoniazid, rifampicin and hexavalent chromium compounds, (M±m)

Group of animals	Age group of animals					
	immature		mature		senior	
	Research duration, days					
	7th	14th	7th	14th	7th	14th
intact control, n=6	12.19±0.55		12.49±0.47		15.09±0.78	
affected by potassium dichromate, n=6	14.82± 0.98	17.97± 1.13*	14.10± 0.91	16.53± 1.17*	19.25± 1.27*	20.97± 1.61*
affected by isoniazid and rifampicin, n=6	13.38± 0.69	18.68± 1.50*	16.53± 1.17*	17.53± 1.61*	18.97± 1.12*	19.25± 1.27*
affected by potassium dichromate, isoniazid and rifampicin, n=6	16.97± 1.62*	23.53± 1.61*	23.10± 1.55*	25.67± 1.28*	23.82± 1.09*	28.08± 1.44*

by 13% and 20% higher than in the immature and senior animals, respectively.

The results of the research on the bile acids content in blood serum of the rats of all age groups are presented in table 5. In animals, which potassium dichromate was administrated to, the content of bile acids increased in all age groups ($p \leq 0.05$). On the 7th day of the research this rate increased by 35% in the immature animals, by 53% – in the mature animals and by 57% – in the senior animals if compared with the animals of the intact control. During the investigation of blood serum of the animals, which anti-TB drugs were administrated to, we evidenced a significant increase in bile acids content in the immature animals in 1.42 and 1.71 times on the 7th and 14th days of the research, respectively.

On the 7th day in the mature and the senior animals the bile acids content increased in 1.54 and 1.76 times; on the 14th day – in 1.50 and 1.80 times, respectively, in comparison with the animals of the intact control.

Discussion

It is established, that aminotransferases catalyze the reaction of transamination between the amino- and α-keto acids, take part in synthesis and forming of body proteins. The increasing activity of blood plasma enzymes, such as ALT and AST, demonstrates the level of hepatocytes damage and indicates liver disorders. Due to this thesis, we studied aminotransferase rate in blood serum and liver of the rats of different age groups affected by xenobiotics. In the liver of the rats after combined adminis-

Table 5. Bile acids content in blood serum (g/L) of rats affected by isoniazid, rifampicin and hexavalent chromium compounds, (M±m)

Group of animals	Age group of animals					
	immature		mature		senior	
	Research duration, days					
	7th	14th	7th	14th	7th	14th
intact control, n=6	6.95±0.43		9.48±0.58		12.02±0.64	
affected by potassium dichromate, n=6	9.36± 0.68*	11.16± 0.62*	14.50± 0.50*	16.37± 0.67*	18.33± 0.98*	20.90± 0.95*
affected by isoniazid and rifampicin, n=6	9.90± 0.49*	11.89± 0.55*	14.60± 0.81*	16.69± 0.65*	18.09± 0.87*	21.59± 0.84*
affected by potassium dichromate, isoniazid and rifampicin, n=6	14.26± 0.55*	16.61± 0.43*	18.23± 0.75*	21.37± 0.87*	22.42± 0.81*	24.16± 0.95*

tration of toxicants we evidenced decrease in this enzyme rate, which indicated the cytolysis of hepatocytes and liver protein synthesis dysfunction in cases of affection by tuberculostatics and hexavalent chromium compounds. It was determined that the increase in ALT rate in blood serum was higher than in liver. It could be caused by toxic influence of potassium dichromate with underlying isoniazid- rifampicin affection of liver. The damaged liver cannot synthesize this enzyme because of hepatocytes damage and their release in blood serum; so we evidenced increased rate of this enzyme.

The determination of aminotransferase rate in blood serum is a sound indicator of the level of pathological process in liver. So we investigated AST rate. AST rate in blood serum increased and was the highest on the 14th day of the research (in 3.7 times higher than the normal range) in the senior animals affected by chrome-isoniazid-rifampicin. The decrease in AST rate in liver proved the transamination of aspartate according to breaking of the Citric acid cycle and negligible release of protein enzyme from tissue cells into blood. It was determined that increase in alkaline phosphatase rate in blood serum of the rats of all age groups after the administration of the investigated toxins into their bodies cause the release of ALP out of the damaged hepatocytes as well as the restoration of its synthesis in bile tubules. We consider that this dynamic activity of alkaline phosphatase may evidence the development of hepatocytes destruction and intrahepatic cholestasis caused by liver architectonics damage and possible development of cirrhosis.

It was established that hepatotoxicity of the metabolites of isoniazid and rifampicin caused the lipid peroxidation of hepatocyte biomembranes and bile formation dysfunction. Rifampicin can also inhibit the glucuronil-transaminases and cause bilirubin metabolism disorders and jaundice [10]. So, we had to examine total bilirubin content in blood serum of the animals affected by isoniazid, rifampicin and hexavalent chromium compounds. Increase in total bilirubin content under the influence of the toxicants evidenced the damage of cell membranes and erythrocytes predominantly and decrease in haemolysis as well as liver excretory dysfunction.

It was established, that in case of drug-induced hepatitis the intestines and liver suffer from the affection, which was caused by the disorders of biosynthesis and hepatoenteral circulation of bile acids. After the combined administration of toxins (potassium dichromate, isoniazid and rifampicin) into the animals' bodies we evidenced that the highest level of bile acids content in immature rats was at the end of the experiment, 239%, in comparison with the animals of the intact control. The increase in bile acids content in blood serum of the affected animals may have the toxic effect on hepatocyte mitochondria that caused increase in ions permeability to internal membrane of mitochondria, ions swelling and release of cytochrome C into cytosol as well as cells apoptosis. The immature animals were the most sensitive to bile formation after administration of tuberculostatics and $K_2Cr_2O_7$, although the increase in bile acids content was evidenced in the mature and senior animals.

Conclusions

We determined the increase in aminotransaminases and alkaline phosphatase rate in blood serum and their decrease in liver. It proved the toxic effect of hexavalent chromium compounds and tuberculostatics on the body of animals of all ages. It caused the disorder of

hepatocyte plasmatic membranes permeability, which was evidenced by significant amount of enzymes in blood and caused liver inflammatory process in the affected animals. So, cholestasis developed. It was characterized by accumulation of bile acids and total bilirubin as well as other bile components in blood that could inhibit the synthesis of components complement in hepatocytes. The most pronounced metabolic disorders in cases of chrome-isoniazid-rifampicin affection were evidenced in body of the animals of immature and senior age in comparison with mature animals.

References

1. Буеверов АО. Лекарственный гепатит: если препарат нельзя отменить. Клинические перспективы гастроэнтерологии и гепатологии 2007; 5: 13–19.

2. Рахимов КД, Пальгова ЛК, Аленова АХ. Справочник по побочным действиям лекарственных средств. Алматы; 2004: 224.

3. Santhosh S, Sini T, Anandan R. Hepatoprotective activity of chitosan against isoniazid and rifampicin-induced toxicity in experimental rats. Eur J Pharmacol 2007; 572: 69–73.

4. Посохова КА, Шевчук ОО, Дацко ТВ. Порівняльна гепатотоксичність антимікобактеріальних засобів та їх комбінацій. Фармакологія та лікарська токсикологія 2010; 5: 41–46.

5. Gliman AG. Antimicrobial agents: drugs used in the chemotherapy of tuberculosis, in Goodman and Gilman's the pharmacologic basis of therapeutics. New York. Pergamon Press; 1990: 1149–1152.

6. Yew WW, Leung CC. Antituberculosis drugs and hepatotoxicity. Respirology 2006; 11: 699–707.

7. Sarich T, Youssefi M, Zhou T. Role of hydrazine in the mechanism of isoniazid hepatotoxicity in rabbits. Arch Toxicol 1996; 70: 835–840.

8. Schwab C, Tuschl H. In vitro studies on the toxicity of isoniazid in different cell lines. Human and Experimental Toxicology 2003; 22: 607–615.

9. Гоженко АИ. Влияния рифампицина на функциональное состояние почек белых крыс. Нефрология 2005; 2: 101–103.

10. Пятночка ІТ, Медвідь ЛІ, Корнага СІ. Гостра нирково-печінкова недостатність – ускладнення лікування рифампіцином. Інфекційні хвороби 2002; 2: 104–106.

11. Трахтенберг ІМ, Короленко ТК, Коршун ММ. Експериментальне вивчення впливу важких металів на організм тварин різних вікових груп. Гигиена труда 2004; 35: 158–170.

12. Duffus JH. Heavy metals – a meaningless term? Pure and Applied Chemistry 2002; 74: 793–807.

13. Recommended health-based limits in occupational exposure to heavy metals. Geneva: WHO, 1980: 205.

14. Hantson P, Caenegem O, Decordier I. Hexavalent chromium ingestion: biological markers of nephrotoxicity and genotoxicity. Clin. toxicol. (Phila) 2005; 43: 111–112.

15. Maeng SH, Chung HW, Kim KJ. Chromosome aberration and lipid peroxidation in chromium-exposed workers. Biomarkers 2004; 9: 418–434.

16. Guide for the care and use of laboratory animals: Eighth edition. The National Academies Press, Washington, DC. – 2011.

17. Reitman S, Frankel S. Definition of biochemical indicators of the toxicity of liver. Amer J Clin. Path 1957; 28: 56–60.

18. Гонський ЯІ, Саяк НП, Рубіна ЛМ. Біологічна хімія. Лабораторний практикум. Тернопіль. Укрмедкнига; 2001: 287.

19. Лапач СН, Чубенко АВ, Бабич ПН. Статистические методы в биологических исследованиях с использованием Excel. Київ. Морион; 2000: 320.

Received: 2015-12-28

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 39–43

DOI 10.11603/ijmmr.2413-6077.2016.1.6380

LEVELS OF NITRIC OXIDE METABOLITES IN RATS WITH HEPATOPULMONARY SYNDROME

I. Ya. Krynytska
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

Background. *System of nitric oxide (NO), which consists of NO, and its metabolites, is very important for various biological processes. NO is signalling molecules and mediators of intracellular and intercellular interaction that causes relaxation of smooth muscles of blood vessel walls, inhibits platelet aggregation and their adherence, is involved in the transmission of nerve impulses, cell proliferation.*

Objective. *The aim of our research was to study the content of nitric oxide metabolites in blood serum and bronchoalveolar lavage, to substantiate their role in pathogenesis of hepatopulmonary syndrome in experiment.*

Methods. *The experiments were performed on 56 outbread male rats, 180-220 g in weight. The first experimental model of hepatopulmonary syndrome (HPS) was made by imposition of double ligature on common bile duct and its further dissection with a scalpel. The second experimental HPS model was made by 8-week intragastric administration of oil solution CCl4 (400 g per 1 L), 0.5 ml per 100 g of body weight on the first day of the experiment, 0.3 ml per 100 g on the third day of the experiment and then every third day until the end of the experiment 0.3 ml per 100 g. A mixture of corn flour, lard and cholesterol and alcohol solution was added to the standard diet of the rats.*

Results. *The total content of nitric oxide metabolites in blood serum of the rats of the experimental group No.1 (on the 31st day after the common bile duct ligation) was significantly increased in 3.9 times (p1<0,001) if compared with the control group №1. In the rats of the 2nd experimental group (with carbon tetrachloride induced cirrhosis) the total content of nitric oxide metabolites in blood serum also significantly increased in 3.1 times (p1<0,001). Comparison of nitric oxide metabolites content in blood serum and bronchoalveolar lavage, which directly indicated about the processes in lung tissue, was great importance.*

Conclusions. *So, in rats with experimental hepatopulmonary syndrome activation of nitroxydergic process by significant increase in nitric oxide metabolites in blood serum and bronchoalveolar lavage took place.*

KEYWORDS: **hepatopulmonary syndrome, nitric oxide metabolites.**

Introduction

System of nitric oxide (NO), which consists of NO, and its metabolites, is very important for various biological processes [2]. NO is signalling molecules and mediators of intracellular and intercellular interaction that causes relaxation of smooth muscles of blood vessel walls, inhibits platelet aggregation and their adherence, is involved in the transmission of nerve impulses, cell proliferation. Cytostatic activity is also presented in NO. Formation of this agent by immunocompetent cells provides protection of body from being infected by bacteria and cancer cells. The researches on participation of NO in the process of apoptosis are very interesting [1, 5, 10]. Contemporary studies on pulmonary disorders are also associated with impaired nitroxidergic dysfunction [3, 7].

NO is a molecule of high reactivity with an effective half-life from 2 to 30 sec, which is formed by the enzymatic oxidation of L-arginine under the influence of cytochrome P-450-like hemoproteins – NO-synthase (NOS). There are 3 isoforms of this enzyme, endothelial (eNOS), neuronal (nNOS) or brain and inducible (iNOS) or macrophagal [4, 6]. As a lipophilic molecule, NO easily diffuses through cell membranes into the neighbouring cells (e.g. from endothelial to myocytes of vessels) where the formed cyclic guanosine monophosphate decreases the level of free calcium and activates the kinase of myosin light chain causing dilatation of vessel [4].

Corresponding author: Inna Krynytska, Department of Clinical and Laboratory Diagnostics, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel.: +3800352254577
E-mail: krynytska@tdmu.edu.ua

Most cytotoxic effects of NO belong to ONOO that is formed in reaction with superoxide. Peroxynitrite is much more active, nitrosates proteins intensively and can be a source of a highly toxic hydroxyl radical in reaction with superoxide anion radical. ONOO- irreversibly inhibits enzymes of respiratory chain nitrosating them and taking iron away. Inhibition of mitochondrial respiration can cause apoptosis [9].

Production of NO by alveoli can influence the hemodynamic and gas exchange in patients with liver cirrhosis. Thus, a direct relationship between alveolar products of NO and hyperdynamic type of circulation was established [12]. Moreover, in experimental liver cirrhosis in rats, hyper-expression of both inducible and constitutional isoforms of NO were observed – synthase in alveolar macrophages and lung endothelial cells [22].

The average life span of nitric oxide in the body is a few seconds. Nitric oxide, which did not participate in chemical reactions, is rapidly oxidized to inactive compounds: nitrites and nitrates. These are nitric oxide stable metabolites, which are the method of this compound synthesis intensity evaluation [18].

So, the aim of our research was to study the content of nitric oxide metabolites in blood serum and bronchoalveolar lavage, to substantiate their role in pathogenesis of hepatopulmonary syndrome in experiment.

Material and Methods

The experiments were performed on 56 outbread male rats, 180–220 g in weight. During the simulation of the pathology 8 animals died. The first experimental model of hepatopulmonary syndrome (HPS) was made by imposition of double ligature on common bile duct and its further dissection with a scalpel. [15] In the control group of animals № 1, common bile duct was separated from the tissue, but not dissected. Postoperative wound was sewed up completely in layers. In the 31st day after the surgery the animals were taken out of experiment under thiopental anaesthesia.

The second experimental HPS model was made by 8-week intragastric administration of oil solution CCl4 (400 g per 1 L), 0.5 ml per 100 g of body weight on the first day of the experiment, 0.3 ml per 100 g on the third day of the experiment and then every third day until the end of the experiment 0.3 ml per 100 g. A mixture of corn flour, lard and cholesterol and alcohol solution was added to the standard diet of the rats. The control group of animals № 2 was on a standard diet of the vivarium and was administered intragastrically the equivalent amount of olive oil. [21].

Animal care and experiments were performed in accordance with the European Convention for the Protection of Animals Used for Experimental and Other Scientific Purposes [14].

Blood serum and bronchoalveolar lavage (BAL) were the subjects of the research.

Quantitative assessment of NO metabolites content was performed by evaluation of their amount, which included nitrite ions that were previously presented in the sample (NO_2^-) and also nitrate ions restored to nitrites (NO_3^-) [2]. Recovery was performed using zinc dust in acidic environment. Nitrites with sulphanilic acid underwent a reaction of diazotization, obtained diazotization solution of N-1 – naftyletylendiamin formed azo dye. Optical density of the obtained colour solution was evaluated by spectrophotometry at absorption maximum and wavelength 536 nm.

According to the evaluation results of calibration solutions optical density (Y), calibration straight line was built and regressor was estimated: Y=A+BX, Y is optical density of calibration solutions; X – concentration of calibration solutions, mmol/l; B – regression coefficient; A – intercept.

The concentration of NO metabolites in the studied sample was estimated by the equation: X1=(Y1–A)/B, Y1 is optical density of the studied sample.

Statistical analysis of the data received was conducted by standard methods of variation statistics using statistical software package. Results are presented as (M±m), M is mean value, m – standard error. Statistical significance of the studied rates was determined by means of paired t-test.

Correlation analysis was performed between the data studied. Linear correlation coefficient (r) and its significance (b) appropriately denoted in the tables (correlation matrices) were evaluated. If index r=0, link was considered to be lost, range 0–0,3 evidenced about weak correlation, index interval 0.3–0.7 demonstrated medium link, and interval 0,7–1,0 proved a significant correlation interaction. The correlation coefficient was significant at $p<0.05$.

Results and Discussion

The total content of nitric oxide metabolites (NO_2^-+NO_3^-) are presented in Table 1.

The total content of nitric oxide metabolites in blood serum of the rats of the experimental

Table 1. Nitric oxide metabolites content in blood serum and bronchoalveolar lavage in rats with experimental hepatopulmonary syndrome (M±m)

Experimental group	Control group № 1 (n=12)	Experimental group № 1 (n=12)	Control group № 2 (n=12)	Experimental group № 2 (n=12)
Blood serum				
NO_2^-+NO_3^-, mcmol/L	36,7±6,0	143,4±14,8 p_1<0,001	33,4±4,4	104,2±9,3 p_1<0,001 p_2<0,05
BAL				
NO_2^-+NO_3^-, mcmol/L	14,1±3,2	81,7±7,6 p_1<0,001	12,0±3,2	54,7±6,9 p_1<0,001 p_2<0,05

Legends:
p_1– significant difference if compared to the control animals;
p_2 – significant difference if compared to the affected animals.

group № 1 (on the 31st day after the common bile duct ligation) was significantly increased in 3.9 times (p_1<0,001) if compared with the control group № 1. In the rats of the 2nd experimental group (with carbon tetrachloride induced cirrhosis) the total content of nitric oxide metabolites in blood serum also significantly increased in 3.1 times (p_1<0,001).

Comparison of nitric oxide metabolites content in blood serum and bronchoalveolar lavage, which directly indicated about the processes in lung tissue, was great importance. It was determined that NO production disorders took place unidirectionally towards the oxidative stress flare. Thus, the total content of nitric oxide metabolites in BAL (Table 1) in the rats of the experimental group № 1 also significantly increased in 5,8 times (p_1<0,001), and in the rats of the experimental group № 2 – in 4.5 times (p_1<0,001).

The correlative analysis showed that, in simulation of hepatopulmonary syndrome by common bile duct ligation, total content of nitric oxide metabolites in blood serum had strong positive correlative link with the content of NO_2^-+NO_3^- in BAL (r=0,87) (p<0,01). In carbon tetrachloride induced cirrhosis (experimental model № 2) the total content of nitric oxide metabolites in blood serum also had a strong positive correlative relationship with the content of NO_2^-+NO_3^- in BAL (r=0,84) (p<0.01). This evidenced the unidirectionality of changes in nitroxydergic processes in blood and lungs in cases of hepatopulmonary syndrome of the applied models.

Probably, the synthesis of nitric oxide in cases of experimental hepatopulmonary syndrome increased due to the activation of inducible NO-synthase under the influence of proinflammatory cytokines and endotoxins, which caused increase in production of NO by liver Kupffer's cells and alveolar macrophages. Our results coincide with the studies of other authors. M. B. Fallon et al. defined and emphasized the role of NO in experimental model of liver cirrhosis, where overexpression of eNOS by pulmonary vessels caused increase in production of endothelin-1 (ET-1) by cholangiocytes, whereby expression of endothelin receptors type B to ET-1 at pulmonary vessels and

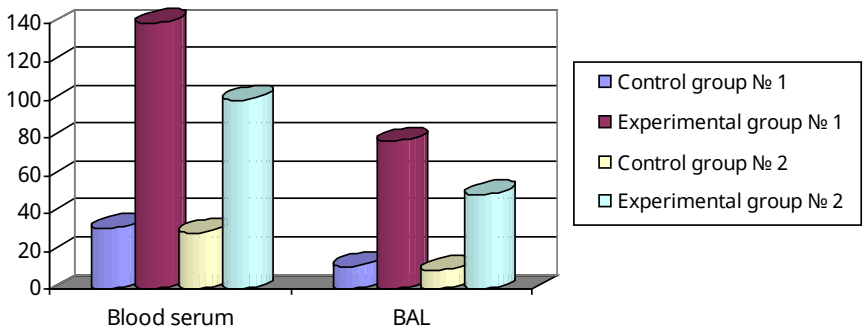

Fig. 1. Comparison of nitric oxide metabolites content in blood serum and bronchoalveolar lavage (* – significant difference if compared to the control animals (p<0,001); # – significant difference if compared to the affected animals (p<0,05).

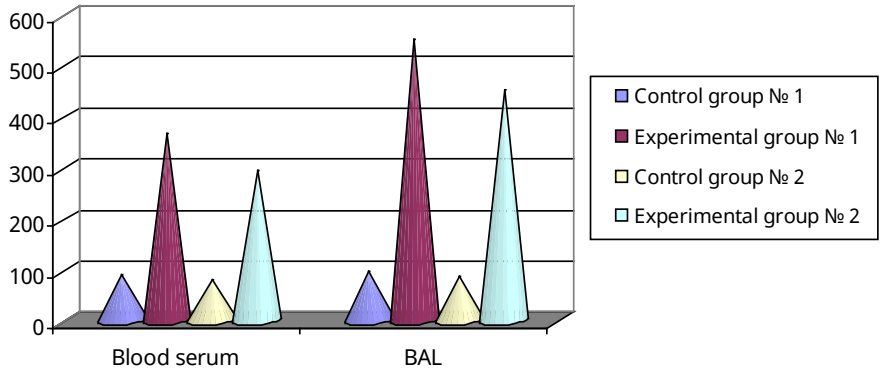

Fig. 2. Comparison of increase in intensity of nitric oxide metabolites content in blood serum and bronchoalveolar lavage (NO_2^-+NO_3^- content in both control groups was equated to 100 %).

synthesis of nitric oxide increased [16]. The level of NO in expired air increased in patients with HPS, and turned to normal in 3–12 months after liver transplantation. [19] Degano B. et al. in a similar study found out that concentration of NO in expired air in patients with liver cirrhosis was 3 times higher than that in the non-cancer [12] By means of the method of flow cytofluorimetery that allows to differentiate alveolar and bronchial origin of NO, the main alveolar increase in formation of NO was determined [13]. It was revealed that NO production by alveoli can influence hemodynamic disturbances and changes in gas exchange in patients with liver cirrhosis. Thus, a close relationship between alveolar production of NO and hyperdynamic circulation type was defined [20]. Moreover, in experimental liver cirrhosis in rats, overexpression of both inducible and constitutional isoforms of NO-synthase in alveolar macrophages and lung endothelial cells was observed [22]. The further studies of NO showed that despite all mentioned above, relationship of NO with portal hypertension, hyperdynamic circulation type and degree of liver damage is unclear. [17] In addition, other molecular mechanisms of vasodilation –nitric oxide independent: enzymatic formation of CO by increase in expression of heme-oxygenase-1, enzymatic formation of H2S and stimulation of calcium-activated potassium channels through endothelial derivative – hyperpolarization factor are described in the literature [8, 11].

Conclusions and Further Research

1. So, in rats with experimental hepatopulmonary syndrome activation of nitroxydergic process by significant increase in nitric oxide metabolites in blood serum and bronchoalveolar lavage took place.

2. After studying the results of nitric oxide metabolites content in blood serum and bronchoalveolar lavage, synchronous development of nitroxydergic processes on systemic and local levels and predominance of nitric oxide synthesis in lungs was determined.

In the future, pro-inflammatory cytokines rate in rats with experimental hepatopulmonary syndrome should be studied for more profound pathogenetic substantiation of nitroxydergic processes intensification.

References

1. Боярчук ОР. Вміст метаболітів оксиду азоту та прозапальних цитокінів у хворих із гострою ревматичною лихоманкою та хронічною ревматичною хворобою серця. Український ревматологічний журнал 2010; 3 (41): 9-13.

2. Козар ВВ, Кудря МЯ, Устенко НВ, Нікішина ЛЕ, Кравченко СВ. Визначення концентрації метаболітів оксиду азоту в сироватці крові. Лабораторна діагностика 2010; 3 (53): 14–16.

3. Марущак МІ. Нітроксидергічні аспекти патогенезу гострого ураження легень в експерименті. Туберкульоз, легеневі хвороби, ВІЛ-інфекція 2011; 3 (6): 69-73.

4. Сапатий АЛ, Купновицька ІГ. Метаболічні особливості оксиду азоту у формуванні ендотеліальної дисфункції за серцево-судинних захворювань. Ліки України 2008; 6 (122): 82–86.

5. Хара МР, Дорохіна АМ. Оксид азоту та серцево-судинна система (огляд літератури). Здобутки клінічної і експериментальної медицини 2010; 1: 14–20.

6. Ячник АІ, Гуменюк МІ, Чопчик АД. Фізіо-

логічні аспекти оксиду азоту при порушеннях легеневого кровообігу та роль L – аргініну в корекції його синтезу. Український пульмонологічний журнал 2008; 1: 40–44.

7. Введенская ЛС, Брегель ЛВ, Горбачев ВИ. Изменения в нитроксидергической системе при легечной гипертензии у детей с врожденными пороками сердца. Педиатрия 2006; 2: 21–24.

8. Гарбузенко ДВ. Патофизиологические механизмы и новые направления терапии портальной гипертензии при циррозе печени. Клинич перспективы гастроэнтерол гепатол 2010; 6: 11–20.

9. Денисенко СВ, Костенко ВА. Изменения митохондриального окисления и фосфорилирования в семенниках белых крыс в условиях избыточного поступления в их организм нитрата натрия. Укр биохим журн 2003; 1: 101–103.

10. Ященко ЮБ, Буряк АГ. Нерешенные вопросы использования оксида азота в качестве маркера диагностики и лечебного средства в неонатологии. Современная педиатрия 2010; 4 (32): 97–100.

11. Carter EP, Sato K, Morio Y, McMurtry IF. Inhibition of K(Ca) channels restores blunted hypoxic pulmonary vasoconstriction in rats with cirrhosis. Am J Physiol Lung Cell Mol Physiol 2000; 279: 903–910.

12. Degano B, Mittaine M, Herve P et al. Nitric oxide production by the alveolar compartment of the lungs in cirrhotic patients. European respiratory Journal 2009; 34(1): 138–144.

13. Delclaux C, Mahut B, Zerah-Lancner F et al. Increased nitric oxide output from alveolar origin during liver cirrhosis versus bronchial source during asthma. Am J Respir Crit Care Med 2002; 165: 332–337.

14. European convention for the protection of vertebrate animals used for experimental and other scientific purposes. Council of Europe Strasbourg 1986; 123: 52.

15. Fallon MB, Abrams GA, McGrath JW et al. Common bile duct ligation in the rat: a model of intrapulmonary vasodilatation and hepatopulmonary syndrome. Am J Phisiol 1997; 272: 779–784.

16. Fallon MB. Mechanisms of pulmonary vascular complications of liver disease. Hepatopulmonary syndrome. J Clin Gastroenterol 2005; 39 (2): 138–142.

17. Gomez FP, Barbera JA, Roca J, Burgos F, Gistau C, Rodriguez-Roisin R. Effects of nebulized N(G)-nitro-L-arginine methyl ester in patients with hepatopulmonary syndrome. Hepatology 2006; 43: 1084–1091.

18. Guevara I, Iwanejko J, Dembinska-Kiec A. Determination of nitrite/nitrate in human biologica materia by he simple Griess reaction. Clin Chim Acta 1998; 274 (2): 177–188.

19. Rolla G, Brussino L, Colagrande P. Exhaled nitric oxide and impaired oxygenation in cirrhotic patients before and after liver transplantation. Ann Intern Med 1998; 129: 375–378.

20. Whittle B, Moncada S. Nitric oxide: the elusive mediator of the hyperdynamic circulation of cirrhosis. Hepatology 1992; 16: 1089–1092.

21. Zhang HY, Han DW, Zhao ZF, Liu MS, Wu YJ, Chen XM. Multiple pathogenic factor-induced complications of cirrhosis in rats: A new model of hepatopulmonary syndrome with intestinal endotoxemia. World J Gastroenterology 2007; 13 (25): 3500–3507.

22. Zhang J, Ling Y, Luo B et al. Analysis of pulmonary heme oxygenase-1 and nitric oxide synthase alterations in experimental hepatopulmonary syndrome. Gastroenterology 2003; 125: 1441–1451.

23. Yaremchuk OZ, Posokhova KA. The liver and kidneys biochemical indices at the experimental pancreatitis in case of the administration of nitric oxide synthesis modulators and recombinant superoxide dismutase. The Ukrainian Biochemical Journal 2011; 83 (4): 57–66.

Received: 2016-02-01

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 44–48

DOI 10.11603/ijmmr.2413-6077.2016.1.6382

MOLECULAR APOPTOSIS MECHANISMS WITH UNDERLYING EXPERIMENTAL ACUTE LUNG INJURY

M. I. Marushchak, I. M. Klishch, Yu. I. Bondarenko, L. P. Mazur
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

Background. *Current data suggest systemic autoimmune activation in the pathogenesis of bronchopulmonary diseases. The imbalance in the system of pro- and anti-inflammatory cytokines is very important in immunopathogenesis.*

Objective. *The aim of our research was to determine the caspase-3 rate in the dynamics of experimental acute lung injury and to study the relationship between their level and the number of cells carrying membrane binding TNF receptor type 1 to define the main mechanisms of cell death.*

Results. *The analysis of the results of caspase-3 rate in lung homogenate showed that this cysteine proteinase was uniformly increasing in all experimental groups during simulating of ALI induced by administration of hydrochloric acid ($p<0.001$). When comparing the results of caspase course of apoptosis it was defined that, despite the progressive increase in caspase-3 rate in lung homogenate, cysteine proteinase rate in plasma did not change.*

The receptor mechanism of apoptosis was studied by establishing correlation relationships with the number of cells carrying membrane binding TNF type 1 (TNF-R1) receptor. A strong positive correlation relationship between the number of neutrophils with TNF-R1 and caspase-3 rate in lungs of all research groups was determined.

Conclusions. *The implementation of neutrophils death by apoptosis is caused by change of activity of caspase cascade effector components, such as caspase-3, in cases of ALI induced by intratracheal administration of hydrochloric acid. One of the potential mechanisms responsible for the activation of caspase course is excessive generation of active forms of oxygen and increase in the number of neutrophils carrying membrane binding TNF receptor type 1.*

KEY WORDS: **caspase-3, tumour necrosis factor alpha receptor 1, acute lung injury**

Introduction

Current data suggest systemic autoimmune activation in the pathogenesis of bronchopulmonary diseases. The imbalance in the system of pro- and anti-inflammatory cytokines is very important in immunopathogenesis. Acute lung injury is manifested by acute inflammatory response in the lung parenchyma that is associated with the severity of damage to the epithelial and endothelial barriers [1]. The latest researches have proved that cytokines, such as tumour necrosis factor alpha (TNF), are the signal molecules of the beginning, development and progression of inflammatory response at local and systemic levels. TNF antagonists are soluble forms sTNF-RI and sTNF-RII, which are formed by separation of active receptor extracellular part from cell membrane [2, 3]. Currently some rather contradictory evidence was published on the effect of cytokines on the programmed cell death. Cytokine rate can be determined by its dose, type of target cells, their functional state and lesions [4].

Two courses of apoptosis are: internal or mitochondrial by Bcl-2 protein family, cytochrome C and caspase-9; and external by caspase-8 activation upon binding of specific Fas cells receptor – and soluble receptors of tumour necrosis factor on the cell surface [5]. Caspases, or cysteine asparagine-protease can be considered a critical effector molecules of programmed cell death, in this case caspase-3 is important for the implementation of both mitochondrial and receptor apoptosis activating [6].

The aim of our research was to determine the caspase-3 rate in the dynamics of experimental acute lung injury and to study the

Corresponding author: Marya Marushchak, Department of Functional Diagnostics and Clinical Pathophysiology, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel.: +380979901202
E-mail: marushchak@tdmu.edu.ua

relationship between their level and the number of cells carrying membrane binding TNF receptor type 1 to define the main mechanisms of cell death.

Materials and Methods

The experiments were performed on 54 white nonlinear mature male rats 200–220g in weight, which were kept on a standard diet at the vivarium of Ternopil State Medical University. The animals were kept and experiments were conducted in accordance with the "European Convention for the Protection of vertebrate animals used for experimental and other scientific purposes" [7]. The animals were divided into 5 groups: the 1st – control group (n=6), the 2nd – animals affected by hydrochloric acid for 2 hours (n=12), the 3rd – animals affected by hydrochloric acid for 6 hours (n=12), the 4th – animals affected by hydrochloric acid for 12 hours (n=12), the 5th – animals affected by hydrochloric acid for 24 hours (n=12).

Anaesthesia for the rats was administrated intraperitoneally with sodium thiopental, 40 mg/kg of animal weight. The ventral side of the neck was treated with chlorhexidine and a 0.5 cm medisection was made to visualize the trachea. Animals were placed in horizontal position at an angle of 45°, HCl, pH 1.2, 1.0 ml/kg was injected by insulin syringe into the trachea at inhale. Physiologic saline, 1.0 ml/kg was administered to the animals of the control group.

In 2, 6, 12 and 24 hours euthanasia was performed for rats by administration of sodium thiopental, 90 mg/kg of the animal weight, following the principles of humane treatment of animals. After their death chest was prosected and cardiopulmonary complex was separated. Heparinized whole blood, lung homogenates and bronchoalveolar lavage (BAL) was used for the research. The standard technique was performed to obtain BAL from lungs [8].

To determine caspase rate in lung homogenate supernatant and leukocyte-lymphocyte blood fractions, 0.25 ml of buffer and 50 mcl of 2 mM DEVD-p-NA was added to 0.7 ml of the test liquid and it was incubated for 2 hours at 37°C; the intensity of light absorbance was measured at 405 Nm, which is directly proportional to the product of hydrolysis of acetyl-Asp-Glu-Val-Asp n-nitroanilide caspase – 3-n-nitroanilide [9].

The number of BAL neutrophils that keep membrane binding TNF receptor type 1 (TNF-R1) was evaluated by the method of flow laser cytometry by means on flow cytometer Epics XL (Beckman Coulter, USA) using radio-labeled monoclonal antibodies to TNF-R1 (CD120a) (Hycult biotech, Netherlands) [10].

Statistical analysis was conducted using the software STATISTICA 6.0. To compare the differences between groups we used t-test in cases of a parametric distribution of alternatives, for calculating other data – one-way ANOVA (Fisher LCD post-hoc test), nonparametric analysis (Mann-Whitney test). The values are presented as Mean±SD, where Mean denotes the mean rate, SD – standard mean error. $P<0.05$ was considered statistically significant.

Correlation analysis was performed between all the studied rates. Coefficient of linear correlation (r) and its fidelity (p) was calculated that was accordingly denoted in the tables (correlation matrices). The correlation coefficient was significant at $p<0.05$.

Results

Caspase-3, which cleaves proteins important for maintaining of cellular homeostasis, is considered to be the main effector molecule of the 'executive' stage in many models of apoptosis. So it was reasonable to determine its rate during apoptosis induced by hydrochloric acid when simulating ALI. Our research on caspase-3 rate showed that the content of this proteinase in blood of the experimental animals suffering from ALI did not change if compared with the data of the control and experimental groups ($p>0.05$) (Table 1).

The analysis of the results of caspase-3 rate in lung homogenate showed that this cysteine proteinase was uniformly increasing in all experimental groups during simulating of ALI induced by administration of hydrochloric acid ($p<0.001$). So, in 2 hours after the beginning of the experiment the caspase-3 rate increased by 49.33% in comparison with the control, in 6 hours – by 26.94% if compared to the second experimental group, in 12 hours – by 23.67% if compared to the third experimental group and in 24 hours – by 28.66% if compared to the previous group (Table 1).

When comparing the results of caspase course of apoptosis it was defined that, despite the progressive increase in caspase-3 rate in lung homogenate, cysteine proteinase rate in plasma did not change. This evidenced the difference in the implementation of programmed cell death, which could be caused by: 1. the varying levels pro-apoptotic signals in blood and lungs; 2. different amount of cells bearing apoptogenic receptors.

Table 1. Rates of caspase-3 in blood plasma and lung homogenate of rats with underlying experimental acute lung injury (M±m)

Rate	Control group n=6	Experimental groups			
		2 n=6	3 n=6	4 n=6	5 n=6
caspase-3, pmol/mg of protein (blood)	19,43±0,88	18,50±1,45	16,65±1,64	15,98±1,41	16,23±1,36
p		p_1>0,05	$p_{1,2}$>0,05	$p_{1,2}$>0,05	$p_{1,2}$>0,05
caspase-3, pmol/mg of protein (BAL)	23,96±4,40	35,78±2,54	45,42±2,72	56,17±3,42	72,27±4,71
p		p_1<0,001	$p_{1,2}$<0,001	$p_{1,2}$<0,001	$p_{1,2}$<0,001

Legends: p_1 – significant difference if compared to the control animals; p_2 – significant difference if compared to the affected animals.

It was established that all populations of white blood cells, which are involved in the inflammatory process of ALI such as neutrophils, secrete cytokines, and vascular endothelium is their main target [11]. The recent researches have proved that cytokines, such as tumour necrosis factor alpha (TNF), are signal molecules of the beginning, development and progression of inflammatory response at local and systemic levels. The bioactivity of TNF depends on the content of cytokine corresponding receptors on the surface of target cells and the number of circulating antagonists [12]. So, the receptor mechanism of apoptosis was studied by establishing correlation relationships with the number of cells carrying membrane binding TNF type 1 (TNF-R1) receptor. A strong positive correlation relationship between the number of neutrophils with TNF-R1 and caspase-3 rate in lungs of all research groups (Table. 2) was determined.

Table 2. Possible relationships between caspase-3 rate and the number of cells carrying membrane binding TNF type 1 receptor in cases of acute lung injury

Rate		Experimental groups	Correlation coefficient, rxy	Correlation relationship probability, p
TNF-R1 rate in ronchoalveolar lavage, %	Caspase-3 rate in lung homogenate, pmol/mg of protein	2	0,88	<0,01
		3	0,90	<0,001
		4	0,95	<0,001
		5	0,81	<0,01

Discussion

A significant increase in caspase-3 rate could be caused by involvement of mitochondrial course of apoptosis, which was associated with the pro-apoptotic signals from inside the cells, such as active forms of oxygen. Previously we proved that intensification of free radical peroxidation processes happened in cases of ALI, and active forms of oxygen were the main cause of that [13]. The generation of oxygen radicals stimulated apoptosis by decrease in mitochondrial membrane potential that verified the mitochondria cell membrane poration and depolarization [14].

Caspase-8, which is activated by the interaction of tumour necrosis factor-α and membrane binding receptor of this interleukin, contribute to pores formation. As a result, mitochondrial matrix swelling developed; internal mitochondrial membrane ruptured; and cytochrome c, AIF (apoptosis inducing factor), which stimulated caspase-3, secondary activator of caspases of mitochondrial origin and other pro-apoptotic proteins released from the intermembranous space into cytosol [15, 16] (Figure 1).

Caspase-3 rate is regulated by both external and internal TNF receptor mediated mechanisms of apoptosis. Currently, it is established that most of the cytotoxic effects of TNF are mediated by TNF-R1 due to its interaction with TRADD (death domains caused by TNF-R1) [17]. Our research also proved it. We evidenced a significant increase in caspase-3 rate with increase in percentage of neutrophils carrying TNF-R1 in cases of ALI induced by intratracheal administration of hydrochloric acid.

Conclusions

The implementation of neutrophils death by apoptosis is caused by change of activity of caspase cascade effector components, such as

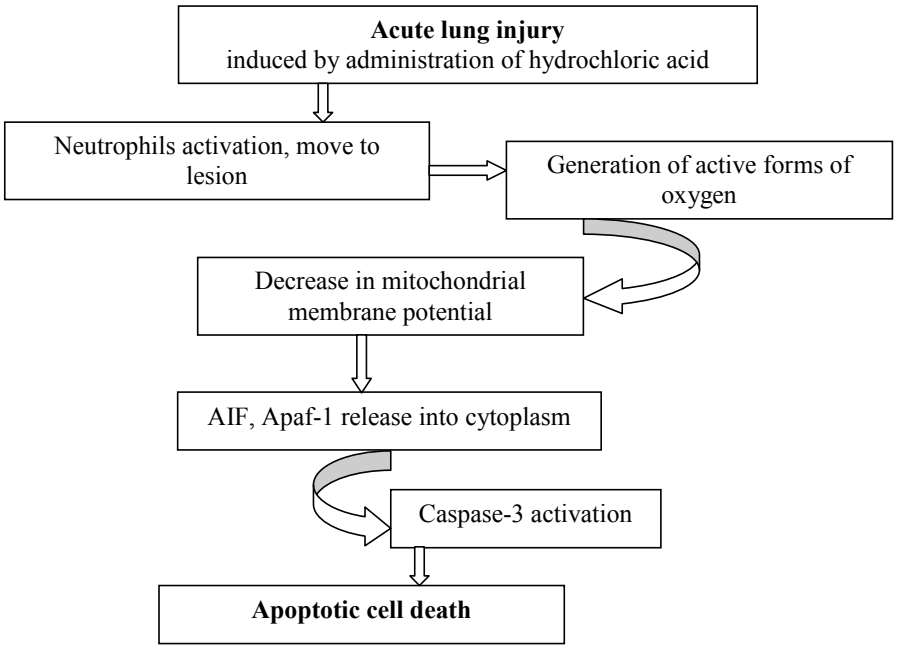

Figure 1. Pathogenic justification of mitochondrial course of apoptosis in cases of acute lung injury.

caspase-3, in cases of ALI induced by intratracheal administration of hydrochloric acid. One of the potential mechanisms responsible for the activation of caspase course is excessive generation of active forms of oxygen and increase in the number of neutrophils carrying membrane binding TNF receptor type 1.

Future Prospects of the Research

In the further research, for pathogenetic study of programmed cell death course we plan to conduct a comparative analysis of the correlation relationships between the early apoptosis level and mitochondrial transmembrane potential rates, active forms of oxygen and caspase rate in blood and bronchoalveolar lavage in rats to detect additional pathogenetic mechanisms of acute lung injury development.

References

1. Mitchell RS, Martin TR. Lung Cytokines and ARDS. Chest 1999; 116: 2–8.

2. Schneider-Brachert W, Tchikov V, Neumeyer J et al. Compartmentalization of TNF receptor 1 signaling; internalized TNF receptosomes as death vesicles. J. Immunity 2004; 21 (3): 415–428.

3. MacEwan DJ. TNF ligand and receptors – a matter of life and death. British Jour. of Pharm 2002; 135: 855–875.

4. Roth Z'graggen B, Tornic J, Müller-Edenborn B et al. Acute lung injury: apoptosis in effector and target cells of the upper and lower airway compartment. Clinical and Experimental Immunology 2010; 161: 324–331.

5. Kaminski M, Kiebling M, Suss D et al. Novel Role for Mitochondria: Protein Kinase Cθ-Dependent Oxidative Signaling Organelles in Activation-Induced T-Cell Death. Mol Cell Biol 2007; 27 (10): 3625-3639.

6. Глумчер ФС, Березняков ИГ, Решедько ГК. 1 Украинский конгресс по вопросам антимикробной терапии: событие для отечественного здравоохранения. Здоров'я України 2007; 2 (1): 16–18.

7. European convention for the protection of vertebrate animals used for experimental and other scientific purposes. Council of Europe. Strasbourg 1986; 123: 52.

8. Гудима АА, Марущак МІ, Габор ГГ, Куліцька МІ. Патогенетична роль нейтрофільних гранулоцитів у розвитку гострого ураження легень. Буковинський медичний вісник 2011; 3: 82–86.

9. Bonomini M, Dottori S, Amoroso L et al. Increased platelet phosphatidylserine exposure and caspase activation in chronic uremia. J Thromb Haemost 2004; 2(8): 1275–1281.

10. Часовских НЮ. Роль протеинкиназ JNK и p38 в регуляции апоптоза мононуклеарных лейкоцитов крови при окислительном стрессе. Бюллетень сибирской медицины 2008; 3: 38–43.

11. Пасечник АВ, Фролов ВА. Апоптоз нейтрофилов как параметр воспалительной реакции

при патологии. Вестник РУДН. Серия Медицина 2004; 25 (1): 103.

12. Mann DL. Recent insights into the role of tumor necrosis factor in the failing heart. Heart Fail Rev 2001; 6(2): 71–80.

13. Грищук ЛА, Марущак МІ. Динаміка перекисного окиснення ліпідів та антиоксидантного захисту в щурів за умов гострого ураження легень. Туберкульоз, легеневі хвороби, ВІЛ-інфекція 2011; 2 (05): 16–20.

14. Мишуніна ТМ, Тронько МД. Основні молекулярні механізми апоптозу та їх порушення при канцерогенезі щитоподібної залози (огляд літератури). Журн АМН України 2006; 12 (4): 611–633.

15. Райхлин НТ, Райхлин АН. Регуляция и проявление апоптоза в физиологических условиях и в опухолях. Вопр онкол 2002; 48 (2): 159–171.

16. Мишуніна ТМ, Калініченко ОВ, Тронько МД, Зурнаджи ЛЮ. Характеристика змін проникності мембран мітохондрій з тканини папілярних карцином щитоподібної залози та з її тканини за інвазії пухлинних клітин. Журн АМН України 2010; 16 (1): 5–22.

17. Chopra M, Reuben JS, Sharma AC. Acute Lung Injury:Apoptosis and Signaling Mechanisms. Experimental Biology and Medicine 2009; 234: 361–371.

Received: 2016-02-02

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 49–53

DOI 10.11603/ijmmr.2413-6077.2016.1.6383

INFLUENCE OF TRIMETAZIDINE METABOLIC THERAPY ON CONNECTIVE TISSUE METABOLISM IN EXPERIMENTAL DIFFUSE ISCHEMIC NECROTIC CARDIOSCLEROSIS IN RATS WITH DIFFERENT RATES OF HYPOXIA RESISTANCE

H. S. Saturska, Yu. I. Bondarenko, U. V. Saturska
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY, TERNOPIL, UKRAINE

Background. *The change in metabolism of the connective tissue elements of heart is the central chain in pathogenesis of diffuse ischemic necrotic cardiosclerosis (DINC), which occurs after repeated epinephrine injury of myocardial tissues.*

Objective. *This study proves that trimetazidine (TM) metabolic therapy has a protective effect on the development of DINC in rats with different rates of hypoxia resistance.*

Methods. *Male white rats were divided into three groups due to the different rates of hypoxia resistance by means of the method of hypobaric hypoxia: rats with low, middle and high rates of hypoxia resistance. Each group was divided into equal subgroups: a control group, a DINC group (injections of epinephrine hydrotartrate (0,5 mg/kg of body weight) and calcium gluconate (5 mg/kg of body weight) two times), a control group administrated with trimetazidine dihydrochloride (10 mg/kg of body weight), a DINC group treated with TM every day (10 mg/kg of body weight) for all period of observation. Concentration of protein-bound oxyproline in blood serum was evaluated on the 7th, 14th and 30th days after the pathology simulation. Histological examination of Masson trichrome staining of myocardium was performed on the 30th days after the pathology simulation.*

Results. *DINC increased the concentration of protein-bound oxyproline in blood serum on the 7th, 14th and 30th days after the pathology simulation, and followed by metabolic imbalances in diffuse connective tissue elements, which are rich in collagens. DINC+TM increased the concentration of protein-bound oxyproline in blood serum less intensively.*

Conclusions. *The intensity of metabolic imbalances in diffuse connective tissue elements is the highest in the low resistant animals to hypoxia. Those results are confirmed by histological examination of the myocardium of rats with different resistance to hypoxia. Fibrotic regions in myocardium are rich in collagens. It has been revealed that the most pronounced therapeutic effect of TM is observed in animals with low resistance to hypoxia, slight – in animals with medium resistance to hypoxia, and the lowest – in animals with high resistance to hypoxia.*

KEY WORDS: **hypoxia, heart, diffuse cardiosclerosis, trimetazidine, oxyproline.**

Introduction

The pathology of the cardiovascular system is the major medical and social problem, because it takes the main cause of morbidity and mortality [1-2]. The special attention is paid to the research on diagnostic markers of degradation and reparation of myocardial tissue [3-5], which would reflect the dynamic changes in myocardium and were predictors of diffuse cardiosclerosis [2-3]. The purpose of this investigation was to determine the changes in the content of protein-bound oxyproline in blood as a diagnostic marker of metabolic activity of collagen at the experimental diffuse ischemic necrotic cardiosclerosis in rats with different rate of hypoxia resistance.

Recently, we demonstrated that the use of trimetazidine as an endogenous cardioprotection inducer in the development of diffuse ischemic necrotic cardiosclerosis is manifested by decrease in manifestations of oxidative and nitrooxidative stress, optimization of immune and cytokine response, stabilization of humoral immune responsiveness [6]. The research was carried out to study the effects of TM on the improvement of connective tissue elements metabolism, indicating inhibition of cardiosclerotic process.

Address for correspondence: Hanna Saturska, Department of Social Medicine, Health Care Management, Economy and Medical Statistics, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel. +380352527233
E-mail: saturska@tdmu.edu.ua

Materials and Methods

Animals and treatment

Experiments were done on 192 male white rats (190-250 g) of the vivarium of Ternopil State Medical University, Ukraine. All animals received care in compliance with the "Guide for Care and Use of Laboratory Animals" (National Institute of Health Publication № 85-23, revised 1985). The studies were carried out according to the National Institute of Health Guide for Care and Use of Laboratory Animals and were approved by the local animal protection committee.

The experimental animals were divided into three groups according to different rates of hypoxia resistance by means of the method of hypobaric hypoxia [6] [Berezovskyi, 1975; Markova, 1998]: rats with low, middle and high rates of hypoxia resistance. Each group was divided into equal subgroups: a control group, a diffuse ischemic necrotic cardiosclerosis group (injections of epinephrine hydrotartrate (0,5 mg/kg body weight) and calcium gluconate (5 mg/kg of body weight) two times), a control group administered with trimetazidine dihydrochloride (10 mg/kg of body weight) every day [6], diffuse ischemic necrotic cardiosclerosis group treated with trimetazidine dihydrochloride (10 mg/kg of body weight) every day for all period of observation (n=8 of each group).

Evaluation of protein-bound oxyproline in blood serum

Concentration of protein-bound oxyproline in blood serum was determined biochemically [7] on the 7th, 14th and 30th days after the pathology simulating.

Histopathology study

The tissue from myocardium ventricles was taken on the 30th day after pathology simulation, then put in 10 % neutral-buffered formalin solution for 5 days, embedded in paraffin, and sectioned. Histological examination of Masson trichrome staining of myocardium was performed [8].

Statistical analysis

Statistical analysis was carried out by OriginPro Program. The results were presented as mean±standard deviation. Differences between experimental groups were analyzed with an unpaired two-tailed Student t-test [9]. Values were considered to be statistically significant at $p<0.05$.

Results

Before the DINC simulation the protein-bound oxyproline concentration in blood serum of rats with low hypoxia resistance was 17.8% ($p<0.05$) higher than in blood serum of rats with middle hypoxia resistance (Table 1), oxyproline concentration in blood serum of high hypoxia resistant animals was 21.9% lower ($p<0.05$) than in blood serum of rats with middle hypoxia resistance. After the DINC simulation protein-bound oxyproline concentration in blood serum of rats gradually increased at all groups.

Under the influence of trimetazidine metabolic therapy the changes in protein-bound oxyproline concentrations in blood serum of the animals with low hypoxia resistance were less pronounced (Table 2). Concentration of this metabolite collagen in 7 days after pathology simulation with TM was by 11.0% ($p<0.05$) (Figure 1) lower than in the group of untreated animals at this stage of observation. On the 14th day after DINC simulation with TM, protein-bound oxyproline concentration in blood serum of the rats with low hypoxia resistance was by 25.3% lower ($p<0.001$) than in the untreated animals, and on the 30th day of observation it was by 33.9% ($p<0.001$) lower than in the

Table 1. Protein-bound oxyproline concentration in blood serum in cases of experimental diffuse ischemic necrotic cardiosclerosis (DINC) with innate hypoxia resistance in rats

Hypoxia resistance rate in animals	Control group (n=8)	Stages of DINC observation		
		7 days (n=8)	14 days (n=8)	30 days (n=8)
Low	49,55±0,59 p<0,05	57,45±1,78 p<0,01 p*<0,01	79,15±2,66 p<0,01 p*<0,01	104,84±3,42 p<0,01 p*<0,01
Middle	42,07±1,10	47,92±0,62 p*<0,01	58,24±1,00 p*<0,01	73,38±3,30 p*<0,01
High	34,52±0,92 p<0,05	38,53±0,55 p<0,01 p*<0,05	42,65±1,19 p<0,01 p*<0,01	56,43±2,84 p<0,01 p*<0,01

Notes: $p<0.05$ – significantly different from middle hypoxia resistant animals at all stages of observation; *$p<0.05$ – significantly different from the control group at all stages of observation.

Table 2. Influence of trimetazidine on protein-bound oxyproline concentration in blood serum in cases of experimental diffuse ischemic necrotic cardiosclerosis (DINC) with innate hypoxia resistance in rats

Hypoxia resistance rate in animals	Control TM group (n=8)	Stages of DINC+TM observation		
		7 days (n=8)	14 days (n=8)	30 days (n=8)
Low	45,58±1,51	51,15±1,36 $p^*<0,05$ $p<0,001$	59,09±1,85 $p^*<0,001$ $p<0,001$	69,32±1,86 $p^*<0,001$ $p<0,001$
Middle	40,58±1,83	43,97±1,34	40,58±1,83 $p^*<0,05$	52,72±3,15 $p^*<0,05$
High	34,90±0,96 $p<0,05$	36,33±0,91 $p<0,001$	37,72±1,52 $p<0,001$	34,90±0,96 $p^*<0,05$ $p<0,01$

Notes: p<0.05 – significantly different from the middle hypoxia resistant of animals at all stages of observation;
*p<0.05 – significantly different from the control group at all stages of observation.

untreated animals with low hypoxia resistance at the similar stage of cardiosclerotic process development without any correction.

In blood serum of the rats with middle hypoxia resistance, protein-bound oxyproline concentration on the 7th day after DINC simulation and trimetazidine correction was by 8.3% (p<0.05) (Fig. 1) lower than in the group of untreated animals at this stage of observation. At the next stage of observation, on the 14th day of DINC simulation, protein-bound oxyproline concentration in blood serum of these animals was by 18.6% lower (p<0.001) than in the untreated ones, and on the 30th day of DINC observation – by 28.2% (p<0.001) lower than in the untreated rats.

There was no significantly difference between the treated and untreated animals with high hypoxia resistance on the 7th day of DINC observation. On the 14th day after the pathology simulation, protein-bound oxyproline concentration was lower by 11.6% (p<0.05) than in the group of untreated animals at this stage of observation; and on the 30th day of DINC observation and correction with metabolic therapy, concentration of protein-bound oxyproline in blood serum was 28.4% (p<0.001) lower than in the untreated animals with high hypoxia resistance without any correction.

Histological examination of the myocardium on the 30th day of DINC observation showed that at heart micropreparations of the healthy animals with different rates of hypoxia resistance, connective tissue was observed slightly in the form of thin collagen fibers (Figure 2), but in heart micropreparations of the animals with DINC (Figure 3) focal cardiosclerosis, perivascular sclerosis hyperelasticity of vessels

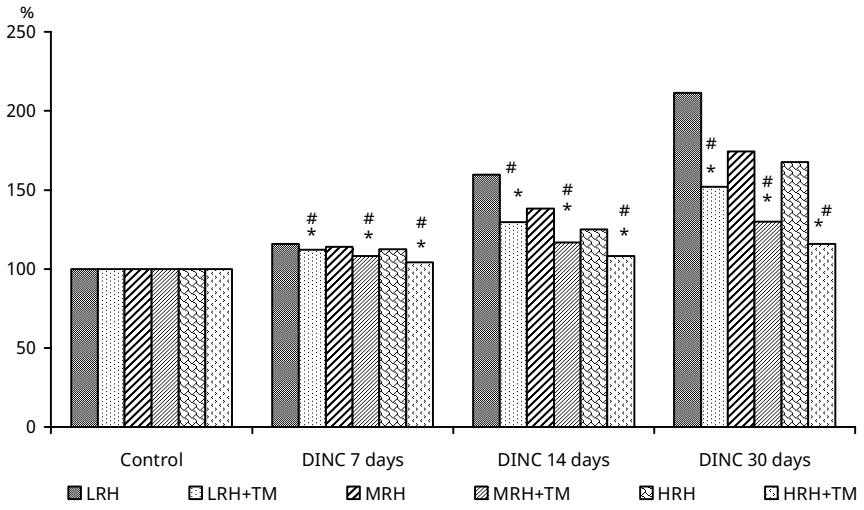

Fig. 1. Influence of trimetazidine on protein-bound oxyproline concentration in blood serum in cases of experimental diffuse ischemic necrotic cardiosclerosis (DINC) with innate hypoxia resistance in rats.
Notes: the indices of the control groups were presented in 100%; * – significantly different from the control group at all stages of observation, p<0.05; # – significantly different from the untreated rats at all stages of observation, p<0.05.

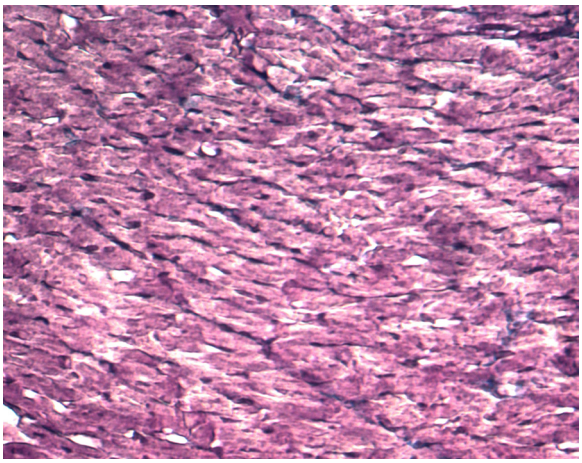

Fig. 2. Myocardium of the control rat. Masson trichrome staining of myocardium. x 400.

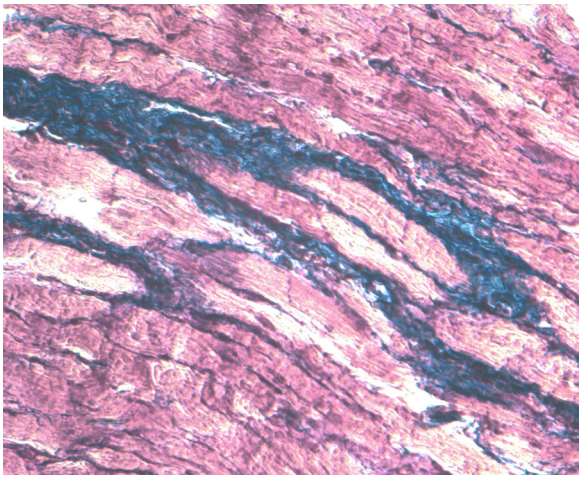

Fig. 3. Myocardium of the low hypoxia resistant rat with DINC. Fibrotic regions in myocardium are rich in collagens and therefore appear in blue upon Masson trichrome staining. In addition, centralized nuclei as well as shape and size distribution of myofibers were detected that was the evidence of pronounced cardiomyocytes hypertrophy. 30-day DINC. Masson trichrome staining of myocardium. x400.

inner membrane, cardiomyocyte hypertrophy, diffuse proliferation of connective tissue were presented. Fibrotic regions in myocardium are rich in collagens and therefore appear in blue upon Masson trichrome staining. In addition, centralized nuclei as well as shape and size distribution of myofibers were detected that was the evidence of pronounced cardiomyocytes hypertrophy.

All the above-mentioned symptoms are the highest in the low hypoxia resistant animals, indicating the intense development of diffuse cardiosclerosis in animals with low hypoxia resistance and confirm the results obtained during evaluation of the concentration of protein-bound oxyproline serum of rats with different rates of hypoxia resistance.

Discussion

Evaluation of protein-bound oxyproline concentrations in blood serum in DINC simulation with and without trimetazidine correction proved that increased collagen production and products of its metabolism [4-5] can be used as a biological marker of the intensity of collagen synthesis in tissue infarction. So we can make the following conclusion: the intensity of metabolic imbalance of connective elements in cases of diffuse ischemic necrotic cardiosclerosis and trimetazidine correction depends on hypoxia resistance of animals. In the low hypoxia resistance animals, maximum effect of trimetazidine correction was manifested; however, more pronounced changes in oxyproline concentration were in DINC simulation without any correction. This effect was not enough for denoting differences between animals with different rates of hypoxia resistance. This matter is characteristic feature of animals with middle hypoxia resistance, but the changes were less pronounced. Animals with high hypoxia resistance were characterized by lower oxyproline concentration, which changed after DINC simulation, so the effect was manifested less, but in general, they are characterized by minimal metabolic disorders of connective tissue elements in the development of DINC and correction with trimetazidine [10-16]. The activity of connective tissue metabolism was studied in experimental diffuse ischemic necrotic cardiosclerosis with different rates hypoxia resistance of a body. The investigations were based on the changes in concentration of protein-bound oxyproline in blood serum that proved adequate metabolic changes in collagen [4–5].

Conclusions

The development of the experimental diffuse ischemic necrotic cardiosclerosis at all stages of observation was accompanied by metabolic imbalance in connective tissue of heart, and was proved by the increase in oxyproline level in blood serum of animals with different rates of hypoxia resistance. The intensity of metabolic imbalances in diffuse connective tissue elements was the highest in low hypoxia resistant animals. Those results were confirmed by histological examination of myocardium of rats with different rates of hypoxia resistance. Fibrotic regions in myocardium are rich in collagens. It has been revealed that the most pronounced therapeutic effect of TM is observed in animals with low hypoxia resistance,

slightly less – in animals with medium hypoxia resistance, and the lowest – in animals with high hypoxia resistance. This matter was evidenced at all stages of observation, but it was the most pronounced in the early period of cardiosclerotic process, indicating the feasibility of early use of metabolic therapy. It explains the absence of cardioprotective effect of trimetazidine in the later stages of cardiosclerosis, when the myocardial fibrosis is already formed.

References

1. Lopez AD, Mathers CD. Measuring the global burden of disease and epidemiological transitions: 2002–2030. Ann Trop Med Parasitol. 2006; 100(5–6): 481–499.

2. Salemi VM, Leite JJ, Picard MH et al. Echocardiographic predictors of functional capacity in endomyocardial fibrosis patients. Eur J Echocardiogr 2009; 10(3): 400–405.

3. Iglezias SD, Benvenuti LA, Calabrese F. et al. Endomyocardial fibrosis: pathological and molecular findings of surgically resected ventricular endomyocardium. Virchows Arch 2008; 453(3); 233–241.

4. Ito A, Yamagiwa H, Sasaki RJ. Effects of aging on hydroxyproline in human heart muscle. Am Geriatr Soc. 1980; 28(9): 398–404.

5. Hoerstrup SP, Zünd G, Ye Q, et al. Tissue engineering of a bioprosthetic heart valve: stimulation of extracellular matrix assessed byhydroxyproline assay. ASAIO J. 1999; 45(5): 397–402.

6. Saturska HS. Peculiarities of cardioprotective effect of trimetazidine at experimental cardiosclerosis in rats with different sensitivity to hypoxia. Vestnik of Vitebsk State Medical University 2015; 14(1): 34–40. (in Russian).

7. Sharaev PN. Method for determination of free and bound hydroxyproline in serum. Lab business 1981; 5: 283–285. (in Russian).

8. Merkulov GA. Course of histological techniques. – L . : Medicine, 1969; 422 p. (in Russian).

9. Orlov AI. Mathematics cases: probability and statistics – the basic facts: a tutorial. M. : M-Press, 2004; 100 p. (in Russian).

10. Detry JM, Sellier P, Pennaforte S, et al. Trimetazidine: a new concept in the treatment of angina: comparison with propranolol in patients with stable angina. Trimetazidine European Multicenter Study Group. Br J Clin Pharmacol 1994; 37: 279–288.

11. Gupta R, Sawhney JP, Narain VS. Treatment of stable angina pectoris with trimetazidine modified release in Indian primary-care practice. Am J Cardiovasc Drugs. 2005; 5(5): 325–329.

12. Marzilli M, Klein WW. Efficacy and tolerability of trimetazidine in stable angina: a metaanalysis of randomized, double-blind, controlled trials. Coron Artery Dis 2003; 14: 171–179.

13. Sellier P, Broustet JP. Assessment of antiischemic and antianginal effect at trough plasma concentration and safety of trimetazidine MR 35mg in patients with stable angina pectoris: a multicenter, double-blind, placebo-controlled study. Am J Cardiovasc Drugs 2003; 3: 361–369.

14. Szwed H, Sadowski Z, Pachocki R, et al. Antiischaemic efficacy and tolerability of trimetazidine in elderly patients with angina. Clin Drug Invest 2000; 19: 1–8.

15. Szwed H, Sadowski Z, Pachocki R, et al. Combination treatment in stable effort angina using trimetazidine and metoprolol: results of a randomized, double-blind, multicentre study (TRIMPOL II). TRIMetazidine in POLand. Eur Heart J 2001; 22: 2267–2274.

16. Szwed H, Sadowski Z, Pachocki R, et al. The antiischemic effects and tolerability of trimetazidine in coronary diabetic patients: a substudy from TRIMPOL 1. Cardiovasc Drugs Ther 1999; 13: 217–222.

Received: 2016-03-04

International Journal of Medicine and Medical Research
2016, Volume 2, Issue 1, p. 54–58

DOI 10.11603/ijmmr.2413-6077.2016.1.6387

ПАТОГЕНЕТИЧНІ МЕХАНІЗМИ ЛЕГЕНЕВИХ УРАЖЕНЬ

М. І. Марущак, І. Я. Криницька, Г. Г. Габор, О. З. Яремчук
ТЕРНОПІЛЬСЬКИЙ ДЕРЖАВНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ ІМЕНІ І. Я. ГОРБАЧЕВСЬКОГО

Вступ. *На сучасному етапі розвитку медико-біологічної науки увага більшості дослідників прикута до ключових механізмів багатьох захворювань людини, які тісно пов'язані з порушенням клітинної смерті. Однією з провідних причин дисрегуляції танатогенної програми клітин є активація біологічних ефектів фактора некрозу пухлин альфа.*

Мета. *Узагальнити існуючі наукові дані щодо внеску метаболітів кисню і монооксиду нітрогену в механізми легеневого ураження.*

Методи. *Аналіз літературних даних щодо механізму легеневого ураження.*

Результати й висновки. *У зв'язку з вищесказаним, актуальною є проблема розуміння сигналів та механізмів, відповідальних за регулювання процесів вільнорадикального окиснення і системи антиоксидантного захисту, роль про- та протизапальних молекул, вплив активних метаболітів на процеси репарації і виживання клітин респіраторного тракту при гострих ураженнях легень. Розуміння суті даних процесів дозволить поглибити існуючі й отримати нові знання при легеневій патології.*

КЛЮЧОВІ СЛОВА: **легеневе ураження, патогенез.**

Вступ

На сучасному етапі розвитку медико-біологічної науки увага більшості дослідників прикута до ключових механізмів багатьох захворювань людини, які тісно пов'язані з порушенням клітинної смерті. Однією з провідних причин дисрегуляції танатогенної програми клітин є активація біологічних ефектів фактора некрозу пухлин альфа (ФНП-α) [1].

Метою даної роботи було узагальнити існуючі наукові дані щодо внеску метаболітів кисню і монооксиду нітрогену в механізми легеневого ураження.

Проведений аналіз наукових джерел показав, що ФНП-α, або кахектин, – плейотропний прозапальний цитокін з молекулярною масою 17 400 кДа, переважно макрофагального походження, який у сироватці крові людей практично не визначається. ФНП-α може діяти як незалежно, так і разом із широким спектром інших факторів, порушувати фенотип і метаболізм клітин у кожної тканини організму. На даний час ФНП-α визнають центральним медіатором серед широкого спектра фізіологічних та імунологічних функцій. Ця молекула проявляє різні біологічні ефекти, в тому числі індукує цитотоксичні ефекти на ендотеліальні клітини, підсилює до них адгезію нейтрофілів шляхом підвищення вироблення хемокінів та адгезивних молекул, збільшує судинну проникність через активацію нейтрофілів, а також стимулює продукування інших прозапальних цитокінів (інтерлейкінів 1, 6, 8) [2, 3].

Респіраторний відділ легень є важливим компонентом системного гомеостазу, який при багатьох патологічних станах, незважаючи на ряд деструктивних і дистрофічних змін у своєму складі, проявляє широкі компенсаторні властивості. Провідне значення в розвитку ускладнень і летальних наслідків належить тяжким прогресуючим респіраторним порушенням, що є одним із компонентів синдрому поліорганної недостатності [4].

Основні чинники легеневого ураження поділяють на прямі та непрямі. До легеневих факторів належать ті, які безпосередньо уражають легеневий епітелій (віруси, бактерії, гриби, токсичний газ, аспірація шлункового вмісту) [5]. Позалегеневі чинники є більш поширеними і зустрічаються при багатьох захворюваннях (цироз печінки, травма, сепсис, панкреатит) внаслідок циркуляції в крові

Corresponding author: Marya Marushchak, Department of Functional Diagnostics and Clinical Pathophysiology, I. Horbachevsky Ternopil State Medical University, 1 Maidan Voli, Ternopil, Ukraine, 46001
Tel.: +380979901202
E-mail: marushchak@tdmu.edu.ua

системних запальних факторів, які мають пошкоджувальну дію на легені. Так, локальна дія хімічно активного травмувального чинника на легеневу паренхіму зумовлює вибуховий викид біологічно активних речовин, зокрема ФНП-α та інших цитокінів. За умов портальної гіпертензії порушується бар'єрна функція печінки, що зумовлює накопичення в крові ендотоксинів. Ендотоксемія стимулює продукування макрофагами печінки і легень вазоактивних субстанцій (оксиду азоту, ендотеліну-1) та цитокіну макрофагального походження – ФНП-α [6–8]. Отже, в основі респіраторного порушення лежить безпосереднє або опосередковане пошкодження епітеліального й ендотеліального бар'єрів, що зумовлює надмірне виділення медіаторів запалення в кровотік, і, як наслідок, ініціюється, активується і поширюється системна запальна відповідь.

Сироваткові цитокіни, зокрема ФНП-α, у свою чергу, є важливими гуморальними регуляторами апоптозу і можуть контролювати дані процеси на генетично детермінованому рівні, а порушення їх апоптотичної активності може призводити до прогресування патологічних станів [9]. Фундаментальні та клінічні дослідження вказують на унікальну здатність ФНП-α ініціювати і апоптотичну загибель клітин, і некроз клітин паренхіматозних органів за умов гіперпродукування ФНП-α [10].

Метаболізм клітини в більшості випадків залежить від характеру інформації, яку несуть первинні месенджери – цитокіни. ФНП-α, як і IL-1β, активує НАДФН-оксидази нейтрофільних гранулоцитів, які окиснюють НАДФН до $НАДФ^+$ за рахунок відновлення O_2 до супероксидного аніон-радикала. Ферментативна генерація супероксидного аніон-радикала в організмі людини здійснюється також ксантиноксидазою, цитохромом Р450, альдегідоксидазою, ліпоксигеназою, циклооксигеназою нейтрофілів і моноцитів [10, 11]. У результаті виникає так званий кисневий спалах, зумовлений активними формами кисню, серед яких мієлопероксидаза каталізує реакцію утворення гіпохлориту з аніона хлору і пероксиду водню, а також відбувається утворення гідроксильного радикала з пероксиду водню і гіпохлориту за наявності іонів заліза (рис. 1) [12, 13]. Дисмутація су пероксид-аніон-радикалів під дією супероксиддисмутаз у біологічних тканинах спричиняє утворення пероксиду водню, який здатний легко проникати через мембрани клітин [14, 15].

Активні форми кисню продукуються також при активації такого ферменту, як NO-синтаза (NOS). Оксид азоту (NO) відіграє важливу роль у регуляції функцій легень і в патофізіології захворювань системи дихання [16–19]. У легенях NO виробляється під впливом конститутивної NOS (cNOS) в ендотеліальних клітинах легеневої артерії та вени. У ряді клітин, наявних у легенях і здатних виробляти NO, включаючи макрофаги, нейтрофіли, гладкі клітини, ендотеліальні, гладком'язові, епітеліальні клітини та, можливо, клітини інших типів, представлена експресія індуцибельної NOS (iNOS). Дослідження, проведені пізніше, показали, що в дихальних шляхах cNOS характеризується високою гомологічністю до iNOS та міститься в епітеліальних клітинах [17].

Утворення ендогенного NO можна розглядати як механізм, який підтримує сталість стану гладких міоцитів у легеневій циркуляції, і, відповідно, недостатність синтезу або ж вивільнення NO зумовлює надмірну їх проліферацію. Проведені наукові дослідження вказують на те, що в нормальному легеневому руслі NO не лише сприяє адекватній вазодилатації, але й перешкоджає вазоконстрикторній дії гіпоксії та ендотеліну [17, 19]. Втрата ендотелієзалежної релаксації судинами під час гіпоксії спричиняє підвищення їх тонусу, утворення тромбів, порушення мікроциркуляції, що призводить до проліферації гладких міоцитів [7, 8].

Літературні дані останніх років свідчать про те, що при запальних захворюваннях органів дихання зростає утворення NO в епітелії дихальних шляхів людини [16]. Синтез NO підвищується за рахунок активації iNOS під впливом макрофагальних цитокінів, що включають й інші клітинні структури в активне утворення медіаторів запалення, а також ендотоксемії. Запальні медіатори, що виділяються мастоцитами, і гіперпродукування NO сприяють збільшенню проникності судинної стінки з формуванням інтерстиційного та альвеолярного набряку [17].

Метаболізм NO відбувається таким чином (рис. 1). Основний шлях – реакція з гемопротеїнами: клітинні ефекти NO здійснюються при зв'язуванні з гемовмісним ферментом гуанілатциклазою, NO реагує з гемоглобіном еритроцитів з утворенням метгемоглобіну. Внаслідок цього NO перетворюється в іон нітриту (NO_2^-), а за наявності гемового заліза NO_2^- переходить у стабільний іон нітрату (NO_3^-). Також NO при взаємодії з

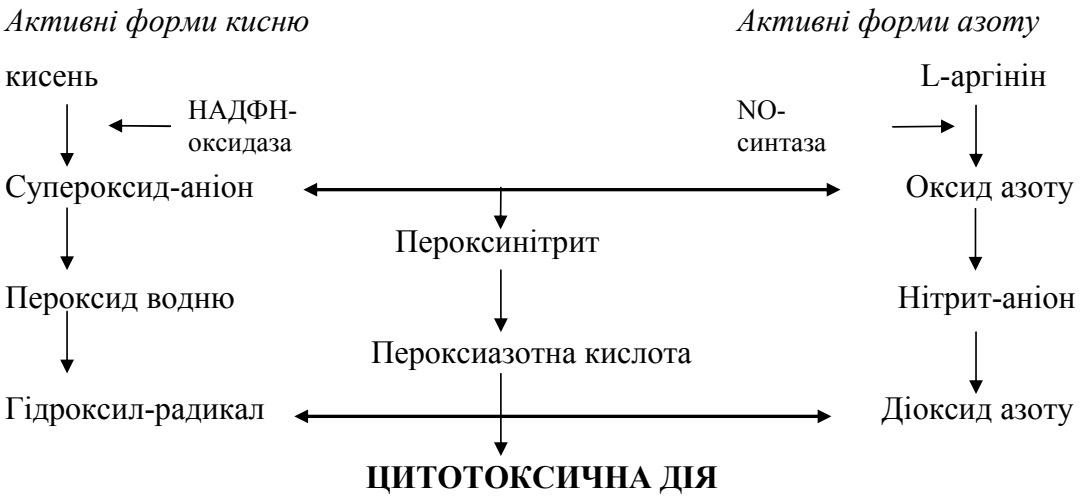

Рис. 1. Шляхи цитотоксичної дії активних форм кисню та азоту.

супероксид-аніоном утворює пероксинітрит і гідроксил-радикал. Утворені сполуки належать до активних форм кисню і мають деструктивну дію відносно білків та ліпідів. Метаболізм NO теж відбувається шляхом утворення нітрозотіолів та динітрозольних комплексів негемового заліза, що є депом NO [18, 19].

Інактивація вільних радикалів здійснюється антиоксидантною системою, яка включає в себе активні антиокиснювачі та антиоксидантні ферменти, що розривають ланцюги молекул під час реакції вільнорадикального окиснення [16]. У надепітеліальній рідині респіраторного тракту є неферментні антиоксиданти: церулоплазмін, трансферин, вітаміни, амінокислоти, іони металів. До основних ферментів антиоксидантної системи, що містяться в надепітеліальній рідині дихальних шляхів, належить супероксиддисмутаза, яка дисмутує супероксидний радикал, глутатіонпероксидазу, що інактивує пероксид ліпідів, каталазу, яка руйнує пероксид водню. Зміни структури і функції субстратів, на які діють активні форми кисню та азоту, залежать загалом від співвідношення активності вільних радикалів та антиоксидантів. Потрібно зауважити, що при гострих легеневих ураженнях антиоксидантні системи швидко виснажуються, а надмірне продукування активних метаболітів кисню може мати руйнівний вплив на епітеліоцити й інші клітини легеневої тканини, що є одним з основних факторів тяжкості захворювань органів дихання [6, 11, 21].

Активні форми кисню та азоту впливають на про- й антиапоптичні механізми клітин респіраторного тракту безпосередньо або через внутрішньоклітинні редоксзалежні сигнальні системи [20, 22]. Так, альвеолоцити I і II типів є чутливими до проапоптичної дії активних метаболітів кисню, які здатні активувати каспазу-3, посилювати експресію проапоптичного протеїну Bax [23, 24]. Монооксид азоту також є регулятором апоптозу, зокрема низька концентрація NO пригнічує, а висока – індукує програмовану клітинну смерть. Пошкодження ДНК активними радикалами азоту зумовлює накопичення p53, що вважають індикатором NO-опосередкованого апоптозу [25, 26].

Висновок

У зв'язку з вищесказаним, актуальною є проблема розуміння сигналів та механізмів, відповідальних за регулювання процесів вільнорадикального окиснення і системи антиоксидантного захисту, роль про- та протизапальних молекул, вплив активних метаболітів на процеси репарації і виживання клітин респіраторного тракту при гострих ураженнях легень. Розуміння суті даних процесів дозволить поглибити існуючі й отримати нові знання, а також намітити подальші напрямки корекції патологічних змін при легеневій патології.

Література

1. Parsons PE, Matthay MA, Ware LB, Eisner MD. National Heart, Lung, Blood Institute Acute Respiratory Distress Syndrome Clinical Trials Network. Elevated plasma levels of soluble TNF receptors are associated with morbidity and mortality in patients with acute lung injury. Am. J. Physiol. Lung Cell. Mol. Physiol. 2005; 288: 426–431.

2. Locksley RM, Killeen N, Lenardo MJ. The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell 2001; 104: 487–501.

3. Lundblad LK, Thompson-Figueroa J, Leclair T. TNF-alpha over-expression in lung disease: a single cause behind a complex phenotype. Am. J. Respir. Crit. Care Med. 2005; 171:1363–1371.

4. Marshall JC. Inflammation, coagulopathy, and the pathogenesis of multiple organ dysfunction syndrome. Crit. Care Med. 2001; 29: S99-S106.

5. Ware LB, Matthay MA. The acute respiratory distress syndrome. N. Engl. J. Med. 2000; 342: 1334–1349.

6. Varghese J, Ilias-basha H, Dhanasekaran R et al. Hepatopulmonary syndrome – past to present. Ann Hepatol. 2007; 6: 135–142.

7. Huffmyer JL, Nemergut EC. Respiratory dysfunction and pulmonary disease in cirrhosis and other hepatic disorders. Respir. Care. 2007; 52: 1030–1036.

8. Zhang HY, Han DW, Wang XC et al. Experimental study on the role of endotoxin in the development of hepatopulmonary syndrome. World J. Gastroenterol. 2005; 11: 567–572.

9. Maianski NA, Maianski AN, Kuijpers TW, Roos D. Apoptosis of neutrophils. Acta Haematol. 2004; 111: 56–66.

10. De Dooy JJ, Mahieu LM, Van Bever HP. The role of inflammation in the development of chronic lung disease in neonates. Eur. J. Pediatr. 2001; 160: 457–463.

11. Lee WL, Downey GP. Neutrophil activation and acute lung injury. Curr. Opin. Crit. Care. 2001; 7: 1–7.

12. Asai T, Ohno Y, Minatoguchi S. The specific free radical scavenger edaravone suppresses bleomycin-induced acute pulmonary injury in rabbits. Clin. Exp. Pharmacol. Physiol. 2007; 34: 22–26.

13.Tamagawa K, Taooka Y, Maeda A. Inhibitory effects of a lecithinized superoxide dismutase on bleomycin-induced pulmonary fibrosis in mice. Am. J. Respir. Crit. Care Med. 2000; 161: 1279–1284.

14. Glosli H, Tronstad KJ, Wergedal H. Human TNF-alpha in transgenic mice induces differential changes in redox status and glutathione-regulating enzymes. FASEB J. 2002; 16: 1450–1452.

15. Ishii Y, Partridge CA, Del Vecchio PJ, Malik AB. Tumor necrosis factor-alpha-mediated decrease in glutathione increases the sensitivity of pulmonary vascular endothelial cells to H2O2. J. Clin. Invest. 1992; 89: 794–802.

16. Shiloh MU, MacMicking JD, Nicholson S. Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase. Immunity. 1999; 10: 29–38.

17. Sittipunt C, Steinberg KP, Ruzinski JT. Nitric oxide and nitrotyrosine in the lungs of patients with acute respiratory distress syndrome. Am. J. Respir. Crit. Care Med. 2001; 163: 503–510.

18. Tracey WR, Xue C, Klinghofer V. Immunochemical detection of inducible NO synthase in human lung. Am. J. Physiol. Lung Cell Mol. Physiol. 1994; 266: 722–727.

19. de Andrade JA, Crow JP, Viera L. Protein nitration, metabolites of reactive nitrogen species, and inflammation in lung allografism. Am. J. Respir. Crit. Care Med. 2000; 161: 2035–2042.

20. Hiwari BS, Belenghi B, Levine A. Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition, and programmed cell death. Plant Physiol. 2002; 128: 1271–1281.

21. Ware LB, Matthay MA. Alveolar fluid clearance is impaired in the majority of patients with acute lung injury and the acute respiratory distress syndrome. Am. J. Respir. Crit. Care Med. 2001; 163: 1376–1383.

22.Melley DD, Evans TW, Quinlan GJ. Redox regulation of neutrophil apoptosis and the systemic inflammatory response syndrome. Clin. Sci. 2005; 108: 413–424.

23. Mikhailov V, Mikhailova M, Degenhardt K. Association of Bax and Bak homo-oligomers in mitochondria. Bax requirement for Bak reorganization and cytochrome c release. J. Biol. Chem. 2003; 278: 5367–5376.

24.Choi IW, Sun-Kim, Kim YS. TNF-alpha induces the late-phase airway hyperresponsiveness and airway inflammation through cytosolic phospholipase A (2) activation. J. Allergy Clin. Immunol. 2005; 116: 537–543.

25. Степовая ЕА, Жаворонок ТВ, Стариков ЮВ. Регуляторная роль оксида азота в апоптозе нейтрофилов. Бюл. эксперим. биологии и медицины. 2008; 146: 646–650.

26. Choi BM, Pae HO, Jang SI, Chung HT. Nitric oxide as a pro-apoptotic as well as anti-apoptotic modulator. J. Biochem. Mol. Biol. 2002; 35: 116–126.

PATHOGENETIC MECHANISMS OF LUNG INJURY

M. I. Marushchak, I. Ya. Krynytska, G. G. Gabor, O. Z. Yaremchuk
I. HORBACHEVSKY TERNOPIL STATE MEDICAL UNIVERSITY

Background. *In contemporary life science research development most attention is paid to mechanisms of many human diseases that are associated with the violation in cell death. One of the main causes of cell thanatologic disregulation program is the changes in their production and activation of the biological effects of tumor alpha necrosis factor.*

Objective *is to summarize current scientific data about role of activated oxygen and nitric metabolites in the system of lung pathogenetic injuries.*

Methods: *analysis of the research data on mechanisms of lung injury.*

Results and conclusions. *The topical issue of lung pathogenetic injury is to understand the signs and mechanisms responsible for regulation of free radical oxidation and antioxidant defense system, the role of pro- and anti-inflammatory molecules, the influence of active metabolites on the process of restoration and survival of the respiratory tract cells in cases of acute lung injury. The studies of this processes will help to obtain more knowledge on lung pathology.*

KEY WORDS: **lung injury, pathogenesis.**

Received: 2016-03-10