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Research Paper

Some Morphometric and Microbiological Aspects of Erosive-Ulcerous Lesions in Upper Part of Digestive Tract in Patients with Hepatocirrhosis and Portal Hypertension.

Ibadov RA, Devyatov AV, Babadjanov A.Kh, Baibekov IM, Irmatov S.Kh, Djumaniyazov DO, Strijkov NA.

J. Life Sci. Biomed., 6 (4): 76-82, 2016;

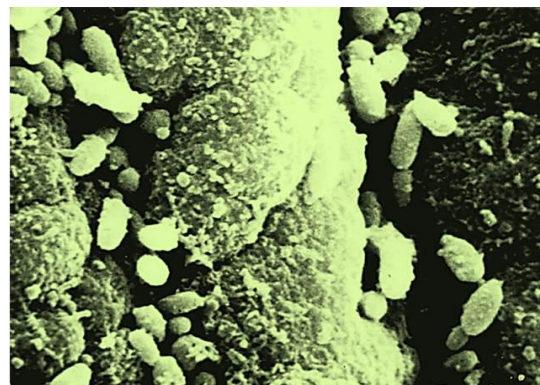
pii:S225199391600013-6

[Abstract]

The present study is directed to the most principal and debatable questions of combined affection of the upper part of digestive tract and liver. Mucosa state of upper part of digestive tract was evaluated on morphometric and microbiological study of digestive tract and liver. Pathogenesis features of the development of erosive-ulcerous process against the background of hepatocirrhosis (HC) progression were characterized. Autopsy with stomach examination was held in 52 patients, which died from HC. Microbiological study was conducted in 40 patients with HC and portal hypertension in admitting to the hospital and after their treatment. As a material for microbiological study we used gastric juice, biopsy materials from mucosa of the esophagus and cardia. Serological study included detection of antibodies (IgG) to *Helicobacter pylori* (Hp). So-called "hepatogenous ulcers" are characterized by specific morphology of atrophic and disseminated with Hp gastric mucosa. HC considerably exacerbates the course of combined erosive-ulcerous affection of esophagus and stomach, which in turn accelerates development of hepatocellular insufficiency. Study shows that so-called "hepatogenous ulcers" have not only specific localization and quantitative evaluation but specific morphologic features that differ them from classic "peptic ulcers".

Author Keywords: Hepatocirrhosis, Hepatogenous Ulcers, *Helicobacter Pylori*

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Research Paper

Determining the Reference Intervals of Long-Chain Fatty Acids, Phytanic Acid and Pristanic Acid for Diagnostics of Peroxisome Disorders in Children.

Mamedov I.S., Zolkina I.V., Glagovsky P.B., Sukhorukov V. S.

J. Life Sci. Biomed., 6 (4): 83-89, 2016;

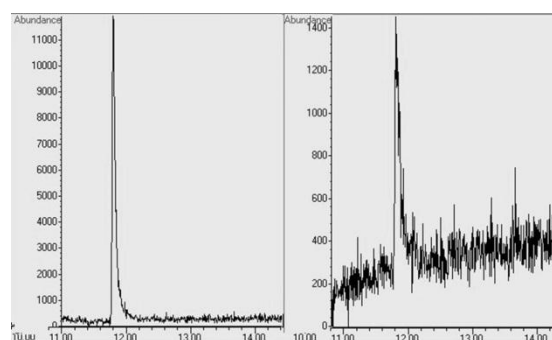
pii:S225199391600014-6

[Abstract]

In this paper we are given the following information about the biochemical properties of long-chain fatty acids (LCFA), phytanic acid and pristanic acids and their biological role. This article describes procedure of the analysis of these compounds by gas chromatography-mass spectrometry (GC-MS) from the sample preparation step to obtain a specific result. Presented reference values of long chain fatty acids and main biochemical markers of peroxisome disorders (phytanic acid and pristanic acid) in plasma and examples described of changes of these values in various pathologies, such as hereditary diseases in peroxisomes in children and adult. The complex GC-MS analysis of LCFA, pristanic acid and phytanic acid is an effective method to identify patients with peroxisome impairment, especially for diagnostics Zellweger syndrome spectrum, rhizomelic chondrodysplasia punctata type 1 and Refsum's disease. This article is intended for doctors clinical and laboratory diagnosis, specialists in the field of clinical genetics, pediatric neurologists, and scientists and audiences.

Author Keywords: Long-Chain Fatty Acids (LCFA), Pristanic Acid, Phytanic Acid, Gas Chromatography-Mass Spectrometry, Peroxisome Disorders

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Research Paper

Characteristic of Shredded Made from Boiled Fish (*Euthynnus Affinis*) with Substitution of Okara.

Yuli Pujiastuti D, Hardoko and Eka Puspitasari Y.

J. Life Sci. Biomed., 6 (4): 90-93, 2016;

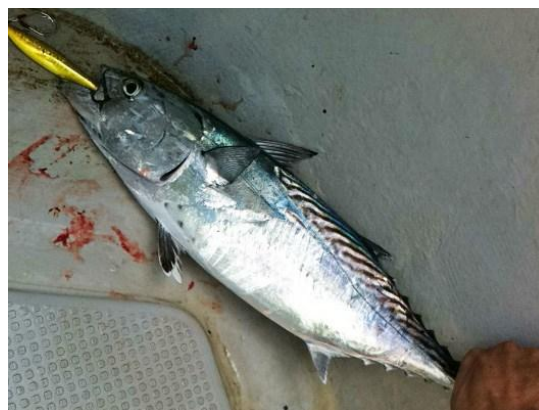
pii:S225199391600015-6

[Abstract]

Boiled fish is one of traditional fish process that people like due to the low price and high nutrient content. However, it has short shelf-life, salty taste and less appearance. Diversification product such as shredded is required to improve the quality of boiled fish. The purpose of this study was to improve the characteristics of shredded made from boiled fish with substitution of okara. Boiled fish was soaked at different water condition (cold and hot) and soaking time (10, 20, 30 and 40 minutes). The data indicated that hot water with soaking time 30 minutes showed higher decreasing salt contents compared with others. The resulting boiled fish was made with substitution of okara (0, 10, 20, 30, 40 and 50%). The results indicated that substitution 20% (w/w) okara showed better characteristic that others with water, protein, fat, ash content, TBA, soluble dietary fiber, insoluble dietary fiber values was 7.60%, 32.08%, 28.72%, 3.23%, 0.27 mg/kg, 1.39% and 7.67%, respectively. Moreover, sensory evaluation was proposed to measure the product's response by panelist and showed significant different in odor and texture

Author Keywords: Boiled fish, Dietary fiber, Okara, Shredded

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Research Paper

Efficacy of Endoscopic Interventions in Prevention of Gastroesophageal Bleeding in Patients with Liver Cirrhosis.

Babadjanov A.Kh. Djumaniyazov D.A., Ruziboev S.A., Baibekov R.R. and Salimov U.R.

J. Life Sci. Biomed., 6 (4): 94-99, 2016;

pii:S225199391600016-6

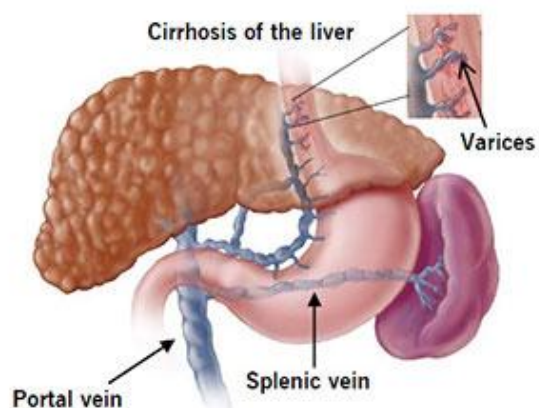
[Abstract]

To analyze the survival of patients with liver cirrhosis, and to assess the effectiveness of endoscopic interventions in the prevention of portal genesis bleedings. To evaluate the efficacy of endoscopic interventions, our study included 449 liver cirrhotic patients with portal hypertension in the period from 1996 to 2015, admitted with bleeding from variceal bleedings or the threat of its recurrence. All patients were divided into 2 groups of the study.

The main group included 239 patients treated between 2008 and 2015 in the control group - 210 patients in the period from 1996 to 2007. The analysis showed that the percentage of patients without recurrence of variceal bleeding when performing only endoscopic interventions was 27% (33 patients) in the control group and 54.2% (64) in the main group. With the phased tactics of portosystemic shunt performance after endoscopic interventions this figure amounted to 32.4% (45) and 109 (61.6%). In the structure of mortality of patients without cirrhosis in the long-term period (81 patients) with endoscopic interventions recurrence of bleeding were observed in 40.7% (33) cases in the control group and 68.1% (64 of 94) in the main group. In turn, when combined endoscopy and portosystemic shunting in the structure of the patients, without counting deaths from progressive liver cirrhosis, the proportion of absence of recurrence was 45.9% (in 45 of 98 patients) and 71.2% (in 102 out of 153 tracked in the remote period excluding deaths from cirrhosis). In the group of patients that do not carry out any endoscopic intervention and the patients received only conservative therapy only in 3 (10.7%) cases it was possible to avoid recurrence of bleeding, which determines the therapeutic ineffectiveness isolated attempts to reduce the risk of recurrence of hemorrhagic syndrome. Modern possibilities of endoscopic technologies have significantly improved the results of treatment and prevention of varicose bleeding or the threat of its recurrence, and the commitment to the phased tactics, with a combination of minimally invasive and traditional decompressive surgery, allowed to increase the survival rates of patients with 80% to 88% - up to 1 year and from 42% to 64% - to 3 years of follow-up.

Author Keywords: Liver Cirrhosis, Portal Hypertension Syndrome, Bleeding From Esophageal And Gastric Varices, Portosystemic Shunting, Endoscopic Ligation, Endoscopic Sclerotherapy.

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Some Morphometric and Microbiological Aspects of Erosive-Ulcerous Lesions in Upper Part of Digestive Tract in Patients with Hepatocirrhosis and Portal Hypertension

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ABSTRACT: The present study is directed to the most principal and debatable questions of combined affection of the upper part of digestive tract and liver. Mucosa state of upper part of digestive tract was evaluated on morphometric and microbiological study of digestive tract and liver. Pathogenesis features of the development of erosive-ulcerous process against the background of hepatocirrhosis (HC) progression were characterized. Autopsy with stomach examination was held in 52 patients, which died from HC. Microbiological study was conducted in 40 patients with HC and portal hypertension in admitting to the hospital and after their treatment. As a material for microbiological study we used gastric juice, biopsy materials from mucosa of the esophagus and cardia. Serological study included detection of antibodies (IgG) to *Helicobacter pylori* (Hp). So-called "hepatogenous ulcers" are characterized by specific morphology of atrophic and disseminated with Hp gastric mucosa. HC considerably exacerbates the course of combined erosive-ulcerous affection of esophagus and stomach, which in turn accelerates development of hepatocellular insufficiency. Study shows that so-called "hepatogenous ulcers" have not only specific localization and quantitative evaluation but specific morphologic features that differ them from classic "peptic ulcers".

Author Keywords: Hepatocirrhosis, Hepatogenous Ulcers, *Helicobacter Pylori*

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INTRODUCTION

Erosive-ulcerous process spreading to the mucosa of cardioesophageal zone, where esophageal and gastric varicose dilated veins (EGVDV) can be located, is one of the detonators that may provoke profuse, often fatal, esophagogastric bleeding. Erosive-ulcerous process in pyloroantral section of the stomach is more often observed in patients with hepatocirrhosis (HC) and portal hypertension (PH); for description of such cases many authors use the term "hepatogenic ulcers" [1].

Since *Helicobacter pylori* (Hp) discovery and admitting its etiological role in the development of stomach (SU) and duodenal ulcer (DU), new data on the influence of this microorganism on pathologic processes in other organs of abdominal cavity, including stomach, have appeared [2, 3]. Recent studies Waluga (2015), have provided evidence that *H. pylori* is also involved in the pathogenesis of some liver diseases. Many observations have proved that Hp infection is important in the development of insulin resistance, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, liver fibrosis and cirrhosis. [4]. Hp DNA was detected in the livers of primary biliary cirrhosis patients [5].

The prevalence of ulcers of the stomach and/or duodenum caused by Hp is higher in patients suffering from hepatic cirrhosis [6, 7]. A recent meta-analysis suggests that there is also a significantly high prevalence of Hp infection among patients with cirrhosis [8]. Eradication therapy may be beneficial for cirrhotic patients because it diminishes the risk of recurrent peptic ulcers and bleeding [9]. However, Stalke et al. [10] demonstrated a positive correlation between the degree of gastric colonization by this bacterium and parenchymatous liver damage in a group of hospitalized patients without liver cirrhosis.

We have not seen serious investigations on pathogenetic mechanisms of Hp influence on the development and course of erosive-ulcerous lesions in upper part of the digestive tract of patients with HC and complications of PH. Taking into account an important role of erosive processes in the development of bleeding from EGVDV, research in this direction is of great importance.

So our study was directed to the main and debatable questions on combined affection of upper part of digestive tract and liver included:

1. Influence of diffuse liver affection on stomach wall;
2. A role of Hp in pathogenesis of gastrointestinal hemorrhages in patients with HC.

MATERIAL AND METHODS

Morphologic research

For studying features of stomach mucosa structure in HC, we examined 52 stomachs of dead patients with HC. Material was fixed in buffered formalin (pH=7,4) and in 2,5% solution of glutaric aldehyde. We used light and scanning microscopy. Morphometry was conducted by projecting-metric method with scale of micrometers (μm).

Microbiological methods

Microbiological study was carried out in admission of patients and after treatment. For microbiological study we used gastric juice, biopsy from mucosa of esophagus and stomach cardia. Gastric juice was received in the morning, on an empty stomach in gastric intubation, biopsy material was taken in esophagogastroduodenoscopy. We identified micro flora in gastric juice (Hp, fungi of the genus *Candida* and other microorganisms), microflora and urease activity were studied in biopsy materials. Isolated microorganisms were identified by generally accepted methods; susceptibility to antimicrobial drugs was tested by disco-diffuse method.

Serological methods

IgG antibodies to Hp was detected by ELISA, test-system ("Hexagon H.Pylori", "Human", Germany). "Hexagon H. pylori" is a qualitative one-step test for determining Hp antibodies in serum or whole blood.

RESULTS AND DISCUSSION

Typical feature of stomach wall in patients with HC is decrease of its thickness in all parts; enlarged sizes of stomach were observed in most cases. In macroscopic examination of structural and functional organization of GM decreases of gastric folds height and their flatness were observed as well as high quantity of viscid mucus. Petechial hemorrhages and erosions were often found in the thickness of mucosa, especially in cardial part. Study of mucosa by scanning electronic microscopy revealed disorders in rhythm conformation as well as significant polymorphism of gastric fossae. Microerosions of different size were a constant sign (Figure 1). Large zones of GM with desquamation of integumentary fossa epithelium. Large quantity of microorganisms were found by this method on the surface of mucosa of gastric fossae, marginal parts of erosions and ulcerations (Figure 2).

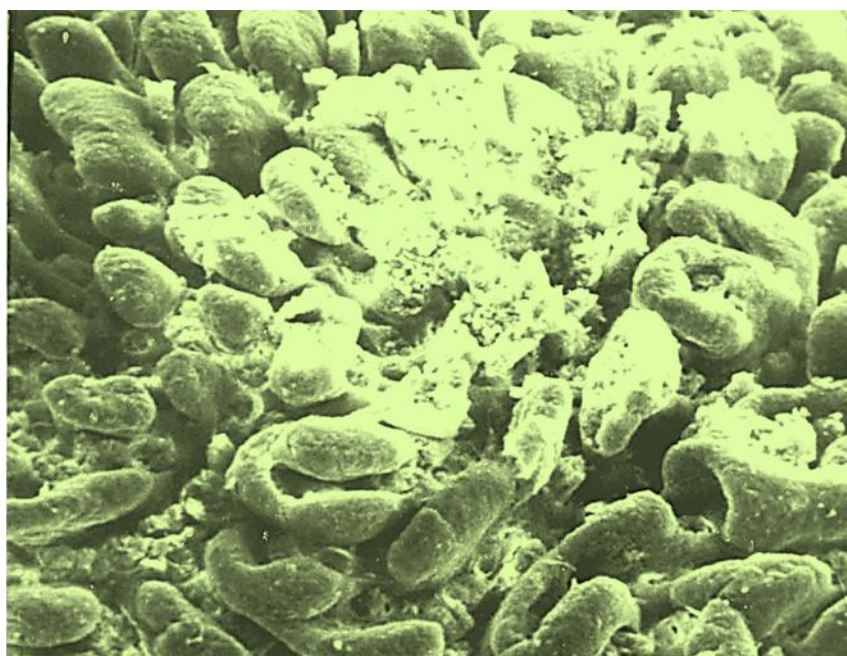


Figure 1. Microerosions of different size were a constant sign SEM x 2000.

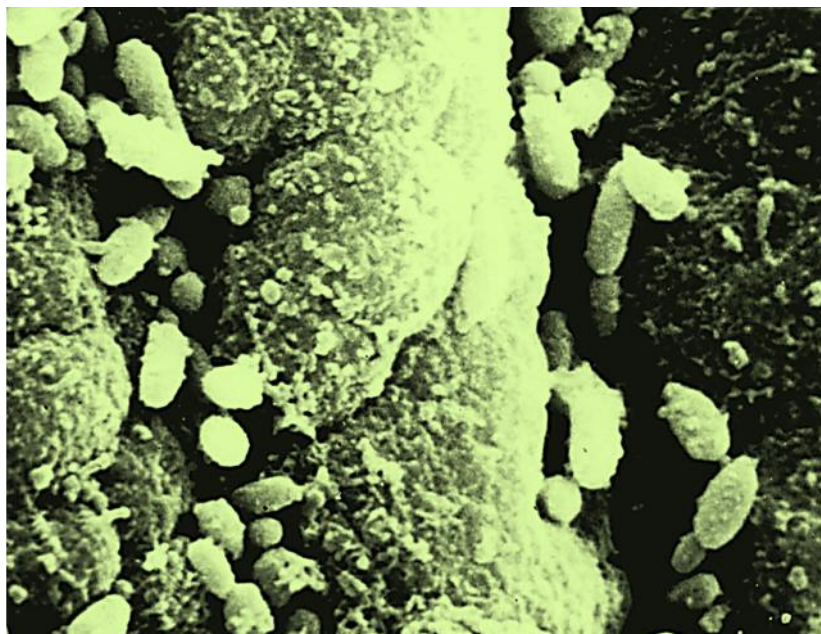


Figure 2. Large quantity of microorganisms were found by this method on the surface of mucosa of gastric fossae, marginal parts of erosions and ulcerations SEM x 10000.

Morphometric study of mucosa showed decreasing of its thickness, most expressed in cardia and minimally in pyloric part. In microscopic study of GM in patients with HC widening and deepening of gastric fossae were observed. The fossae often occupy about 1/3 of GM height, rarely achieve till its center. Surface epithelium is transformed from high prismatic into cubic one. As it was mentioned above its desquamation and denudation of spaces between fossae also take place.

There is a decrease of glands quantity per length unit. The present process is observed both in cardial and fundal parts, its minimal expression can be observed in pyloric part. In all parts interfossal spaces are enlarged at the expense of edema, small thin-walled vessels of vein type are found in quantity as well as considerably enlarged lymphatic fissures. Practically in all cases moderately expressed lymphoid cellular infiltration with increase of intensity in basal parts of GM was observed.

Cardial glands become shorter and straighter; quantity of mucocytes in secretory parts decreases. Parietal cells are not detected in HC. Highly-specialized fundal glands are pathologically changed to the most degree. Quantity of these glands compared to the control samples decreases down to 40, 9 - 3, 2 (70, 3 - 4, 1 in health). Atrophic and dystrophic processes in fundal glands are localized mostly in main and parietal cells, their quantity decrease in comparison to the control samples by 21 and 14,9% respectively. These highly-specialized cells in pathology under investigation are substituted by mucocytes and "pylorisation" of fundal glands takes place. In ultrasound study of cardial glands decrease of size and quantity of mucocytes is observed as well as reduction of amount of mitochondria and granular endoplasmic reticulum in them, diminution of the number of secret granules in apical part of cytoplasm is also detected.

There is also reduction of mitochondria's number in parietal cells which become small, their intracellular channels are reduced, amount of tubulovesicles is decreased. Nuclei are polymorphic, mainly small, with decreasing of chromatin electronic density. Significant quantity of myelin structures occur in the cytoplasm.

The main cells are of small size, their cytoplasm contains a minute quantity of small mitochondria, content of granular endoplasmic reticulum profiles is decreased, reticulum is cistern-like. Quantity of secretory granules is low; they are often of mixed character (mucoidisation). Content of pathologic inclusions and vacuoles is increased with characters of myelin degeneration in mitochondria. Nuclei are of small size, chromatin is significantly clarified. Structures of the lamellar complex are distended by content with low electronic density. Quite a number of parietal and main cells in fundal glands are replacing by mucocytes.

Glands of the pyloric part are affected by atrophy to a less extent, sometimes their moderate proliferation can be observed. Thin-walled vessels of venous type, lymphatic fissure, moderately expressed edema and fibrosis occur in interglandular stroma. Submucous layer of stomach is dilated in patients with HC, complicated by PH, due to expressed edema, various focal or diffuse lymphoid-cellular infiltrates as well as different number of

venous type vessels. These vessels are of various diameter, large veins are often located close to mucosa. Dilatation of lymphatic fissures close to mucosa also can be observed in some cases.

It should be noticed that changes in submucous layer parameters in HC, complicated by PH, are maximally occurred in cardial part and minimal in pyloric part. Venous type vessels of the largest diameter are usually found in cardial part and more rarely in pyloric part. It is also of great importance that changes in submucous layer, described above, depend on a level of vascular system development in the zone of cardioesophageal transition. According to our data muscular layer of stomach in HC is thinned, almost by a factor of two in all cases. This process is more expressed in cardial part and more rarely in pyloric one. The leading sign of decrease of muscular layer thickness is atrophy of smooth muscle cells and their substitution by elements of connective tissue. In this case muscular layer is exposed to fasciculation and fibrosis. Venous type vessels of various diameter and lymphoid-cellular infiltration of various intensity are also found there.

Degree of muscular layer fibrosis directly depends on HC duration and level of PH.

Subserous layer of the organ is also affected by edema and respectively increase in thickness. Fiber structure of the connective tissue is disturbed; a lot of vessels of venous type and different diameter, mostly of small diameter are located there.

Erosion were observed in 22 (42%) of section material, ulcerous lesions of stomach were found in 7 (13,4%).

Thus, our study shows that so-called "hepatogenous ulcers" have not only specific localization and quantitative evaluation but specific morphologic features that differ them from classic "peptic ulcers":

1. Basic number of ulcers were localized in pyloroantral part;
2. In half of the cases ulcers were multiple (2-4);
3. Ulcers were mainly flat, with small depth and flat edges (Figure 3);
4. Fundus of the ulcers was covered with a thin layer of fibrinoid necrosis with a very weak development of granulations (Figure 4);
5. There is a large quantity of venous vessels of various diameters in fibrous layer, often located at a small depth;
6. Signs of ulcer epithelization were absent in almost all the cases;
7. Inflammatory infiltration was present in all the cases.

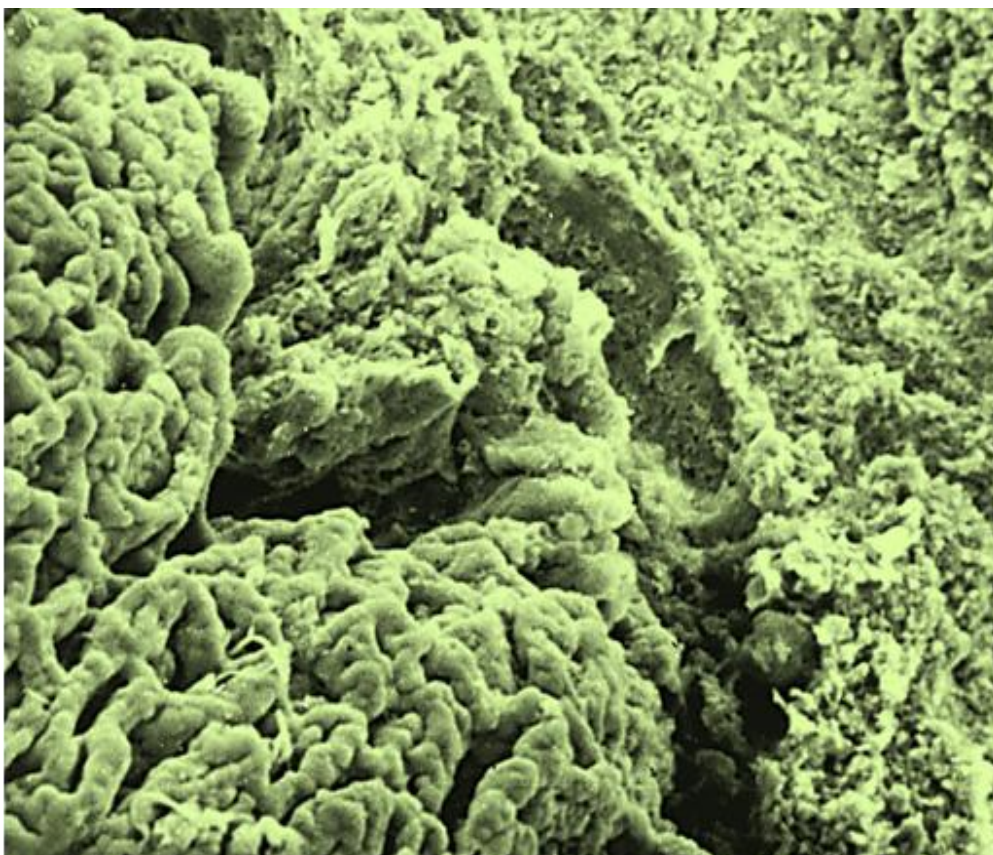


Figure 3. Ulcers were mainly flat, with small depth and flat edges. SEMx60.

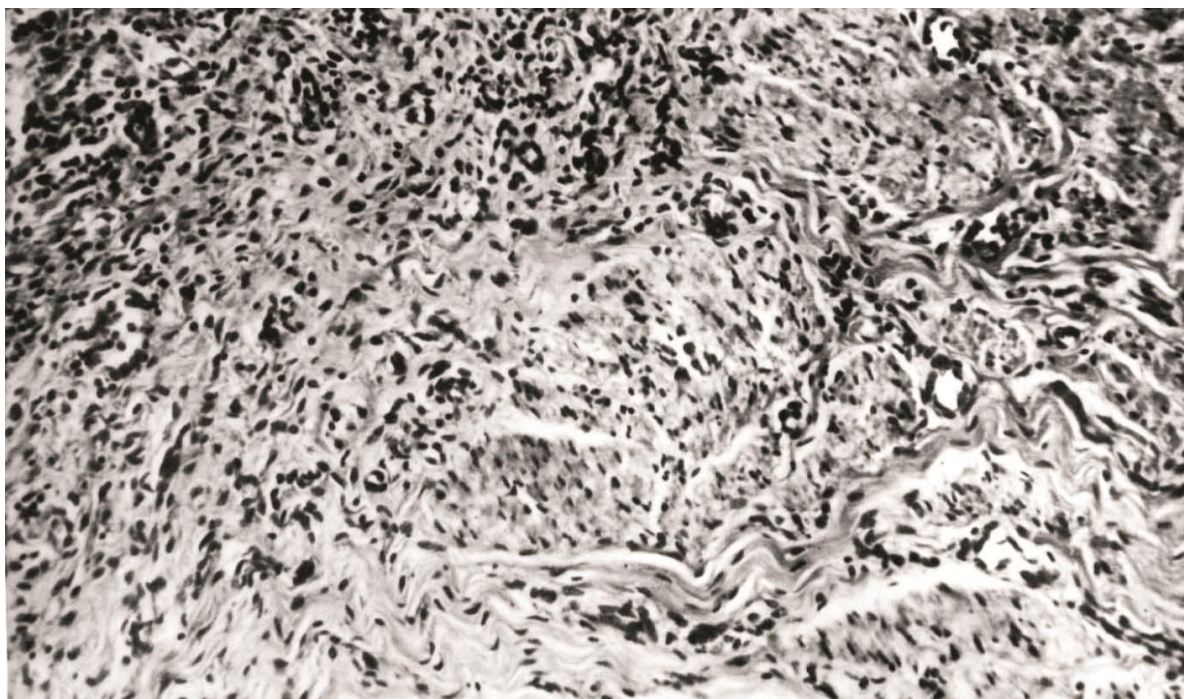


Figure 4. Fundus of the ulcers was covered with a thin layer of fibrinoid necrosis with a very weak development of granulations. LM 10 x 40

Results of our microbiological study are presented in Table 1. The table demonstrates, that Hp in gastric juices persisted in 23 (57,5%) of patients, fungi from *Candida* genus—in 17 (42,5%) of patients, mold fungi in 3 (7.5%), other microorganisms—in 10 (25,0%) of patients. Hp were more often isolated from gastric juice 23 (57.5 %) of patients, then from biopsy material from cardia 9 (45.0%) of patients and esophagus 5 (25.0%) of patients. IgG antibodies to Hp were detected in 10 (66.6%) of patients and it can be considered as evidence of the fact that persistence of Hp is not indifferent to macroorganism and causes appropriate response.

Along with Hp *Candida* were often found in examined patients, indicating to a significant decrease of barrier functions of the stomach and organism as a whole. Study of gastric juices acidity showed that only 9 (22,5%) of patients had normal secretion (BAP - basal acid production 5-10 mmol/hour), decrease of gastric secretion was detected in other 31 (77.5%) of patients (BAP < 5 mmol/hour). Isolation of Hp depending on gastric juice acidity level was of special interest. Analysis (table 2) shows a tendency of Hh isolation as acidity increases and a reverse tendency was detected for *Candida*. This tendency was especially clear in analysis content of Hp and *Candida* content in gastric juice.

One fact also attracts our attention: in all 4 examined patients with bleeding from EVVD and stomach, who came in emergent order, high intensity of Hp infection (10^4 - 10^5 CFU/ml) and expressed serologic response (antibodies against Hp), were observed. However we don't have enough data to make concrete conclusions.

Table 1. The results of our microbiological examination of patients with HC and PH

Nº	Material under study	H.pylori	Candida	Mold fungi	Other microorganisms
1	Gastric juice n=40.	23 (57.5%)	17 (42.5%)	3 (7.5%)	10 (25.0%)
2	Biopsy from gastric cardia.n=20.	9 (45.0%)	13 (65.0%)	0	13(65.0%)
3	Biopsy from esophagus. n=20.	5 (25.0%)	10 (50.0%)	0	10 (50.0%)
4	Blood serum (antibodies to Hp).n=15.	10 (66.6%)	x	x	x

Table 2. Influence of gastric juice acidity on microflora in patients with HC and PH n=40

Acidity level of gastric juice	Number of patients, %	H.pylori		Candida	
		Frequency, %	Contamination CFU/ml M±m	Frequency, %	Contamination CFU/mlM±m
Normal (BAP = 5-10)	9 (22.5%)	6 (66.6%)	$3.9 \times 10^6 \pm 2.1 \times 10^5$	4 (44.4%)	$5.0 \times 10^3 \pm 1.9 \times 10^2$
Reduced (BAP<5)	31 (77.5%)	16 (51.6%)	$3.0 \times 10^4 \pm 1.3 \times 10^3$	21 (67.7%)	$2.0 \times 10^4 \pm 1.1 \times 10^3$
P			<0.001		<0.001
Increased (BAP>10)	0	-	-	-	-

Our data testifies that hypochlorhydria of gastric juice is prevalent in the examined patients, that results in migration of Hp from pyloroantral part of stomach, where acidity is reduced due atrophic processes in mucosa, to upper part of stomach (corpus and cardial part) where they find more appropriate conditions for their vital activity. At the same time reduced gastric acidity favors to Candida development that worsens disbiotic processes in the digestive tract.

DISCUSSION

The relevance of problem erosive-ulcerous lesions in upper part of digestive tract in patients with hepatocirrhosis and portal hypertension in clinical practice is determined by a large number of publications over many years.

Stomach function and secretions are altered significantly in patients with cirrhosis, both with or without portal hypertension motivation by the abnormalities of gastric acid and pepsin secretion, and gastrin release. Histological and endoscopic changes, and the impaired cytoprotection associated with cirrhosis, are discussed in the context of abnormal gastric secretion. In addition, the symptomatology and association of Hp, and treatment of duodenal ulceration in cirrhosis are discussed [11].

The result of many early studies is the conclusion that additional studies are needed to further understand of erosive-ulcerous lesions in upper part of digestive tract in patients with hepatocirrhosis and portal hypertension. Early H. pylori eradication is associated with a lower risk of recurrent peptic ulcers in cirrhotic patients. H. pylori eradication is the mainstay for treating cirrhotic patients who have contracted peptic ulcers [12].

So high frequency of helicobacteriosis in patients with HC and PH may be caused by various general and local factors: total decrease of resistance to infection in patients with HC, disorders in blood circulation in portal system, contributing to local decrease of resistance in mucosa of gastroduodenal zone. In turn Hp invasion worsens all the processes described above, causing progressive mucosa atrophy and development of hypo- and achlorhydria. It is also necessary to remember that various strains of Hp differ by their virulence, i.e. ability for adhesion on GM and, probably, esophagus, and some other pathogenic features that allow Hp to affect tissues of macroorganism up to development of bleeding and other complications. This is confirmed by the studies of Wen et al. [13] H. pylori infection impairs the expressions and functional activities of duodenal mucosal bicarbonate transport proteins, CFTR and SLC26A6, which contributes to the development of duodenal ulcer [13].

Due to unique mobility and some other factors, Hp and products of their metabolism may penetrate into esophagus and manifest pathogenic properties, negatively influencing changed mucosa over EVVGD.

It would be wrong to ignore a question about biological properties of isolated strains of Hp as a possible etiological agent of pathologic processes in mucosa of the gastroduodenal zone and esophagus in patient with HC and PH, however such data were presented in study in our center. The results of the study indicated to the presence of eubiotic drugs characterizing by significant antagonistic activity against Hp cultures, isolated from patients with HC and PH. The author described a group of specific preparations, which can be used in the complex treatment of such patients. Besides correction of intestinal dysbiosis, these preparations exert antagonistic action, direct and indirect, on Hp, persisting in an organism.

Walton [14] in one of the recent issues learn about the virulence factors that have made Helicobacter pylori such a successful pathogen in hepatocirrhosis, when it focuses on in vitro findings that may shed light on epithelial-mesenchymal transition that occurs during the process of fibrosis [14].

Thus, Hp are frequently found in patients with HC and PH and worsen the course of the main disease, contributing to the development of hemorrhages from EVVGD. Pathologic process in these patients lasts for a long time, and they admitted to hospital with advanced cases with considerable atrophy of mucosa and respectively essentially reduced secretory activity; but at the initial phases of the process these symptoms are manifested weaker. Hp infection is much intensive, frequency of the presence of antibodies to Hp is higher than isolation of Hp from stomach, i.e. due to the development of some negative for Hp conditions, microorganisms are eliminated to some extent, but antibodies against Hp continue to circulate in patients' blood.

CONCLUSIONS

1. Distinctive features of so-called "hepatogenous ulcers" are not only their specific localization and quantitative presentation but also some definite morphologic properties: Multiple flat ulcers in pyloroantral part covered with a thin layer of fibrinoid necrosis with a weak development of granulations and number of venous vessels of various diameter in fibrous layer;
2. "Hepatogenous" erosive-ulcerous process in stomach of patients

with HC and PH lasts for a long time with a significant atrophy of mucosa and, respectively, low secretory activity, against background of HP colonization of high intensity, worsening the course of the main disease and contributing to the development of esophageal and gastric hemorrhages.

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Competing interests

The authors declare that they have no competing interests.

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Determining the Reference Intervals of Long-Chain Fatty Acids, Phytanic Acid and Pristanic Acid for Diagnostics of Peroxisome Disorders in Children

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ABSTRACT: In this paper we are given the following information about the biochemical properties of long-chain fatty acids (LCFA), phytanic acid and pristanic acids and their biological role. This article describes procedure of the analysis of these compounds by gas chromatography-mass spectrometry (GC-MS) from the sample preparation step to obtain a specific result. Presented reference values of long chain fatty acids and main biochemical markers of peroxisome disorders (phytanic acid and pristanic acid) in plasma and examples described of changes of these values in various pathologies, such as hereditary diseases in peroxisomes in children and adult. The complex GC-MS analysis of LCFA, pristanic acid and phytanic acid is an effective method to identify patients with peroxisome impairment, especially for diagnostics Zellweger syndrome spectrum, rhizomelic chondrodysplasia punctata type 1 and Refsum's disease. This article is intended for doctors clinical and laboratory diagnosis, specialists in the field of clinical genetics, pediatric neurologists, and scientists and audiences.

Author Keywords: Long-Chain Fatty Acids (LCFA), Pristanic Acid, Phytanic Acid, Gas Chromatography-Mass-Spectrometry, Peroxisome Disorders

Abbreviations: GC/MS: gas chromatography-mass-spectrometry; PUSFA: polyunsaturated fatty acids; DNA: deoxyribonucleic acid

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INTRODUCTION

The introduction of the achievements of molecular genetics, immunology, analytical biochemistry, morphology, and other sciences has led to undifferentiated state of a whole class of new diseases, related to change the structure and function of intracellular structures - lysosomes, mitochondria, peroxisomes, the so-called "diseases of cell organelles".

Due to the structure and function subcellular structures at different human pathology it was became possible isolate mitochondrial disease, lysosomal diseases, peroxisomal diseases. However, if the study of the first two classes of diseases is progressing significantly, the study peroxisomal diseases are not given enough attention [1, 2]. Peroxisomes play an important role in the catabolism of polyamines processes "peroxisome breathing», (β -oxidation of very long chain - 24-26 carbon atoms or more) and dicarboxylic fatty acids (C26 and above). These acids usually ingested in the diet and, because they are not part of the human lipid shall be destroyed. These related, in particular, phytanic acid, contained in plants. Oxidation of very long chain fatty acid with peroxisome enzyme acyl-CoA oxidase going in the liver, adipose tissue, kidney, intestines, lung, spleen, adrenals. In peroxisomes observed degradation some xenobiotics containing acetalphosphatides and catabolism of prostaglandins.

An important role is played by peroxisomes in the synthesis of certain vital components necessary for the body, for example, plasmalogens. These phospholipids in which the fatty acid is not combined with glycerin ester (enol) and aldehyde bond. They constitute from 5 to 20% of phospholipids and membranes necessary for formation of nervous tissue. Plasmalogen protect cells from oxygen free radicals. Peroxisomes are involved in transamination of glyoxylate, which are formed with the participation of glycolate oxidase and peroxisomal may further metabolized to oxalic acid. Alanine glyoxylate aminotransferase hereditary enzyme deficiency in liver peroxisomes leads to the development of hyperoxaluria type I, since glyoxylate thus converted into oxalic acid.

Due to the variety of functions peroxisome becomes apparent that a violation of one or more metabolic functions may cause peroxisomal disorders [2]. Such disorders typically result in accumulation in tissues and

biological fluids of one, several or all of the respective metabolites, depending on the number of functional disorders. These savings are used for (differential) diagnosis of peroxisomal biochemical disorders accompanied by the absence or dysfunction of peroxisomes. Diagnosis is particularly important in identifying peroxisomal disorders in children, because, for example, with Zellweger syndrome, if the detection of the early stages does not occur, children die a few months after birth from severe hypotension, eating disorders, convulsions, seizures of liver and heart.

Peroxisomes or microbodies, are widely represented in human cells of all tissues except erythrocytes. They are a round or oval formations with diameter ranging from 0.2-1 μm (in liver and kidneys) to 0.1-0.2 micrometers (as amniocytes and fibroblasts). The peroxisomes, there are about 40 types of enzymes, takes an important part in the oxidative metabolism of cells, metabolism of bile acids, fatty acids, cholesterol, gluconeogenesis [3]. Peroxisomes play an important role in protecting cells from forming in their matrix of atomic oxygen (result of hydrogen peroxide decomposition) [2]. Part of peroxisome oxygen absorption is approximately 20% from summary oxygen consumption in the liver. Peroxisome enzymes use oxygen to oxidize various substrates, producing hydrogen peroxide. Excess hydrogen peroxide can be dangerous for the cells, however, thanks to the presence of enzyme - catalase, quickly decomposing hydrogen peroxide, prevented damage to cells, and the presence of superoxide dismutase which protect cell from another toxic compound of oxygen - superoxide anion.

A recent study have shown that peroxisomes derived from a special subdomain of the endoplasmic reticulum and, therefore, do not have their own DNA, are semi-autonomous organelles that are able to grow and is divided into subsidiaries peroxisomes. It is now widely known that peroxisomes catalyze a number of important metabolic functions that can not be achieved by other organelles.

From the standpoint of human genetic diseases, of particular interest are the following processes: 1) beta-oxidation of fatty acids; 2) biosynthesis of lipid; 3) alpha-oxidation of fatty acids and; 4) glyoxylate detoxification. To accomplish this, a set of functions, peroxisomes have a unique set of enzymatic proteins that catalyze different reactions. Besides, the membranes have a system of selective peroxisome transport for transferring substrates from the cytosol into the organelles and outputting its end products of metabolism.

So far, there is no uniform classification of peroxisomal disorders. This is due to the small study the function of peroxisomes and the lack of a single criterion, which could form the basis of the classification. Attempts to use to justify the classification of morphological criteria (presence or absence of peroxisomes in the cells) were unsuccessful. In recent years there has been research to use as fundamental criteria peroxisomal disorders primary biochemical and genetic defects.

To date, the foundation of separation peroxisomal disorders based on two criteria - morphological (presence or absence of peroxisomes in the liver) and biochemical (violation of one or of several functions of peroxisomes), which must be assessed in each case at the same time. This allows you to identify three groups of peroxisomal disorders:

Group 1 - disorders associated with generalized violation of the biological functions of peroxisomes and the absence or significant decrease in the number of peroxisomes in the liver. This class includes syndrome Zellweger (SC), the infantile form of Refsum's disease (IRD), neonatal adrenoleukodystrophy (NALD), point osteochondrodystrophy, some forms of Leber's congenital amaurosis, rhizomelic chondrodysplasia punctata Type 1 (RCDP1), and others. For those diseases characterized by complete violation of the biogenesis of the peroxisomes, but to varying degrees. When RCDP first type biogenesis peroxisome broken partially and syndrome Zellweger mainly violated all peroxisome function, resulting in the accumulation of a number of peroxisomal metabolites in the plasma, whereas RCDP first type affected only biosynthesis lipids and alpha oxidation phytanic acid [4, 5].

Group 2 - disorders caused by violation of several biological functions of peroxisomes in the normal number of peroxisomes in the liver. These include the syndrome of pseudo Zellweger, D-bifunctional protein deficiency [6], Selwaganapathy syndrome etc.

Group 3 - includes disorders in which damaged the biological function of peroxisomes and there is a normal content of peroxisomes in the liver. This group is also divided into different subgroups, including disorders peroxisome beta-oxidation - X-linked adrenoleukodystrophy (X-ALD), deficiency of acyl-CoA-oxidase 1 [7], failure 2-methyl-acyl-CoA reductase (MACoAR), deficiency of the protein transporting styrene (STB) [8], violations biosynthesis of lipids (failure dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase) [9], violations of the alpha-oxidation of phytanic acid (Refsum's disease, adult type) [10] and, as the sole representative, violation of glyoxylate detoxification with hyperoxaluria first type, caused by lack of alanine aminotransferase glyoxylate [11].

Table 1 shows the various peroxisomal disorders and levels of LCFA, pristanic acid and phytanic acids for each of these disorders. The data indicate that the content of LCFA increased in the spectrum disorders Zellweger, and when X-ALD, deficiency of acyl-CoA oxidase and failure D-bifunctional protein, but normally in the case of other disorders including the deficit of the STB and the deficit MACoAR. However, if the last two violations accumulated pristanic acid and bile acids are intermediates di- and trihydroxychalcone acids. Pristanic acid level increases when the in the spectrum disorders Zellweger, at RCDP first type and Refsum's disease [10].

Table 1. Levels of long-chain fatty acids, pristanic acid and phytanic acid in various peroxisomal disorders.

Group 1	Long chain fatty acids	Pristanic acid	Phytanic acid
Spectrum of disorders Zellweger	↑	N-↑*	N-↑*
Rhizomelic chondrodysplasia punctata Type 1 (RCDP1)	N	↓-N	N-↑*
Group 2			
Peroxisome disorders of β-oxidation			
X-linked adrenoleukodystrophy (X-ALD)	↑	N	N
Deficiency of acyl-CoA-oxidase	↑	N	N
Deficiency of D-bifunctional protein	↑	N-↑*	N-↑*
Deficiency of the protein transporting styrene (STB)	N	N-↑*	N-↑*
Deficiency of 2-methyl-acyl-CoA reductase (MACoAR)	N	N-↑*	N-↑*
Disorders of the biosynthesis of lipids			
Rhizomelic chondrodysplasia punctata Type 2	N	N	N
Rhizomelic chondrodysplasia punctata Type 3	N	N	N
Violations of the alpha-oxidation of phytanic acid			
Refsum disease	N	N	N-↑*
Violation of detoxification of glyoxylate			
Hyperoxaluria First type	N	N	N

N = normal level; ↑ = higher level; * = Levels can range from normal to high, depending on the power and age

In order to use specific markers for the diagnosis of various metabolic disorders must be defined reference intervals in large healthy populations of different ages, nationalities and both genders. And though health care professionals understand the importance of reference intervals, many laboratories still do not have their own reference range, especially for the pediatric population.

The properties of the test substances

Long chain fatty acids as well as the fatty acid with a branched chain: pristanic acid and phytanic acid are extremely hydrophobic and practically insoluble in water. Inside the cell, they are in the form of esters of coenzyme A. These acids are generally present in lipid-tissues such as adipose tissue, but also, they can be components of various physiologically important lipids such as myelin. In this regard, LCFA and fatty acids with a branched structure are abundantly present in many tissues and organs. LCFA have a cyclic and branched structure exist in the form of esters such as triglycerides, phospholipids, cholesterol esters, or even in the form of carnitine esters. In the free form LCFA difficult to detect because, bind to plasma proteins, such as albumin. Consequently, LCFA not filtered by the kidneys and not be present in urine [13]. LCFA and saturated fatty acids with a branched structure are stable compounds, they are not destroyed in the presence of oxidants. In this regard, for the storage of samples is sufficient to freeze them. Although patients with abnormal peroxisome function will be observed an increased content LCFA with chain that longer than 26 carbon atoms [4], in the process of diagnosis can also use fatty acid: hexacosanoic acid C26:0, lignoceric acid C24:0, behenic acid C22:0, and their relations [12].

MATERIALS AND METHODS

The study included 168 healthy children aged 2 to 16 years who were passed routine medical inspection of Science research clinical institute of pediatrics. Pre from parents of patients was obtained written informed consent for the study. For analysis were collected from 2 to 5 ml of venous blood. As preferably used

anticoagulant EDTA, centrifuged for 10 minutes, then the plasma was collected by aspiration, and stored at – 200 °C. If possible, blood samples should be done before breakfast, directly after sleep. However, since the level of LCFA shows only minimal daily variations are also suitable samples taken in the afternoon. Importantly, because the content long chain fatty acids, phytanic acid and pristanic acids does not change during storage of samples at room temperature for several days, the samples can be transported under these conditions, although we recommend them to freeze, especially if the transit time exceeds 48 hours. The level of content of the test compounds in frozen plasma is kept unchanged for 2 years. In our work we used the method of gas chromatography with mass detection (GC-MS) and electron impact ionization. Determination of content long chain fatty acids, phytanic and pristanic acids was performed by gas chromatography with mass spectrometric detection firm Shimadzu GCMS QP-5050A, AOC-equipped autoinjector -20i. The method involves the preliminary derivatization of N-methyl-N-(tert-butyltrimethylsilyl)trifluoroacetamide (MTBSTFA) in combination with the use of stable isotopes for the fatty acids: hexacosanoic acid C26:0, lignoceric acid C24:0, behenic acid C22:0, and pristanic acid (3, 7, 11, 15 tetramethylhexadecanoic acid) and phytanic acid (2, 6, 10, 14 tetramethylpentadecanoic acid) [14, 15]. In order to determine the total content of LCFA, pristanic acid and phytanic acid samples should be subjected to acid and alkaline hydrolysis followed by extraction with hexane.

A large number of plasma samples taken from different patients, were combined and thoroughly mixed. Of the total mixture aliquoted into eppendorf with 150 µl and stored at - 20 °C. For each series of analysis used samples derived from a mixture of plasma sample one (pool).

RESULTS

Analysis chromatograms of the blood of patients, obtained by GC-MS from children with peroxisomal disorders and chromatograms of blood from children in the control group shows differences that can even evaluate visually, without quantifying processing the received data. A detailed analysis of chromatograms noteworthy increase in peak marker metabolites which are indicative of at respective pathologies. Thus, Figure 1 shows a chromatogram of patient with identified pathologies and patient from the control group.

The reference values were defined as confidence interval 2,5-97,5% spread in the control group. Reference values of unbranched fatty acids were obtained by analyzing the 168 control samples by GC/MS (Table 2).

Pathological values may differ for different inherited disorders peroxisomal functions. It is essential to link the cases with the maximum number peroxisome functions. Selective screening peroxisome violations in our lab can include an analysis of how LCFA, phytanic acid, pristanic acid, and pipecolate bile acids in plasma and in erythrocytes plasmagene.

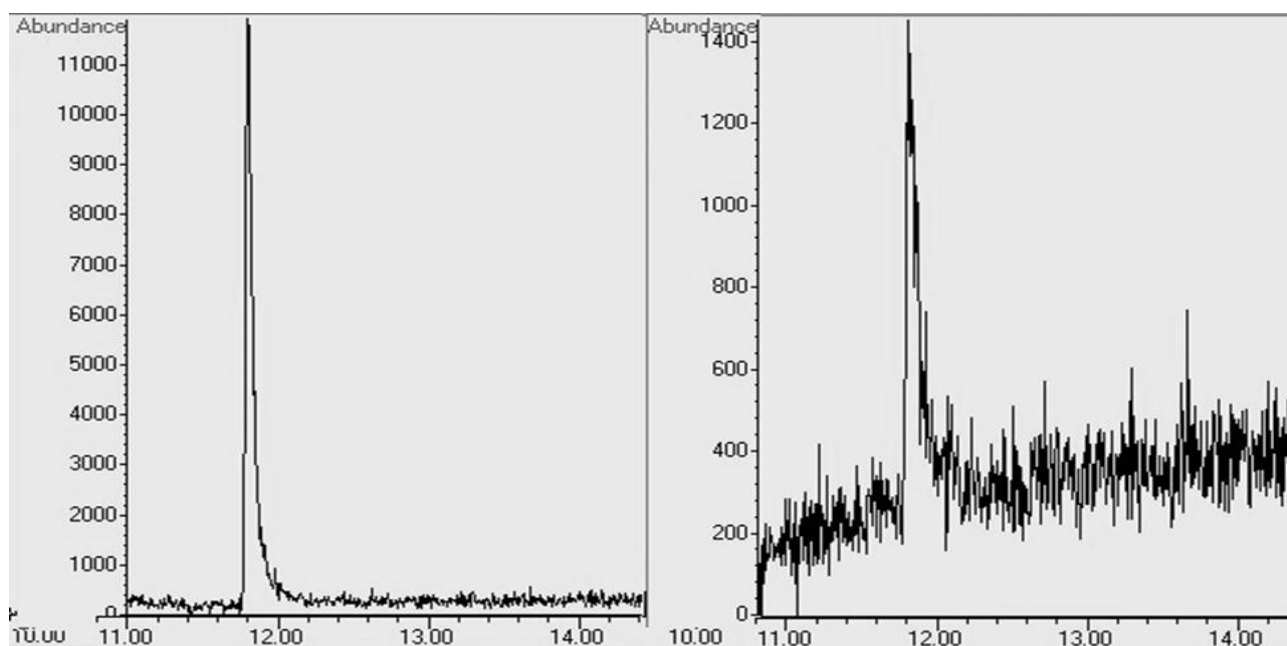


Figure 1. Comparison of typical chromatograms blood samples from a patient with Refsum's disease (marker - phytanic acid) and the patient from control group. The X-axis - time of chromatography (min), Y-axis- intensity of signal in absolute units.

Table 2. Concentrations of long chain fatty acids plasma control samples ($\mu\text{mol/L}$).

Detection compound	Mean value	2,5-97,5% confidence interval
Behenic acid C22:0	77	42-121
Lignoceric acid C24:0	54	31-86
Hexacosanoic acid C26:0	0,78	0,45-1,34
C24/C22	0,76	0,56-0,94
C26/C22	0,01	0,01-0,02
Pristanic acid	1.52	0-3,4
Phytanic acid	3.26	0,92-8,5

DISCUSSION

At this moment in the literature is very little published research results with those obtained reference intervals of biochemical markers of peroxisome diseases both in children and in adult populations. The obtained data in this study are consistent with previously published [18, 19].

Long chain fatty acids synthesized in peroxisomes. These cell organelles not exhibit appreciable changes in protein activity during the day or with age. In this connection, reference values can be determined rather precisely. As it has already been described in earlier studies, long chain fatty acids concentration in control group is independent of age [15, 18]. Our findings can be applied to children with hypotonia and characteristic dysmorphic symptoms of the syndrome of Zellweger, but also adults (both men and women) with unexplained leukodystrophies. Probably, for adult patients would be limited to long chain fatty acids analysis aimed at the identification of X-linked adrenoleukodystrophy or adrenomyeloneuropathy (AMN), but recently published data on failure MACoAR and protein STB indicate the need for wider screening, because these patients equally possible flow and other metabolic processes [8].

With regard to pathological values, the most informative is a fatty acid C26:0. Most patients with the syndrome of Zellweger the content of fatty acid C26:0 is equal to 3-12 $\mu\text{mol/L}$, which exceeds the reference value of 3 to 10 times. For comparison, in men with the disease X-linked adrenomyeloneuropathy adrenoleukodystrophy and the levels of C26:0 in mostly 2-4 $\mu\text{mol/L}$. False-negative results for men with these diseases are extremely rare, in contrast to the levels of C26:0 in women, patients with X-linked adrenoleukodystrophy, which varies from 1.1 to 2.9 $\mu\text{mol/L}$ and, thus, coincides with the normal. In the case of fatty acids C24:0, the situation is slightly different: there is a significant overlap between the levels of this acid in patients and normal control sample. However, the ratio of C24:C22 with the value for control sample $< 0,92$ is excessive for almost all patients and is equal to 1.06, but in women with X-ALD, the ratio of C24:C22 may be equal to up to 0.8. One analysis of long chain fatty acids not sufficient to completely exclude X-ALD; accurate test result in this disease can only be obtained if this analysis is accompanied by DNA analysis. In patients with hereditary disorders of peroxisomal function, induced by increased levels of fatty acids like C26:0 and the ratio of C24:C22. The increase in the levels of C26:0 rarely leads to the correct diagnosis. However, the constant deviation level of long chain fatty acids and/or their ratio should be checked when studying fibroblasts, in order to properly diagnose and recommend genetic testing of the family. False-positive level of long chain fatty acids is rare; the only well-known example is the ketogenic diet (a diet high in fat and low amount of carbohydrates), therefore, for the correct conclusion and the diagnosis must take into account the results of the analysis of the content at the same time of pristanic and phytanic acids. As a rule, in patients with impaired biogenesis of the peroxisome, or violations in the system peroxisomal β -oxidation show increased levels of both fatty acids with a branched structure in different ratios.

The exception, of course, are patients with ALD/AMN or deficiency of acyl-CoA oxidase, having a normal level of branched fatty acids. Patients with Refsum's disease can have extremely high levels of phytanic acid, to 1500 $\mu\text{mol/L}$, and very low levels of pristanic acid ($< 1 \mu\text{mol/L}$) due to a deficiency of phytanoyl-CoA-hydrolase. A less pronounced increase in the level of phytanic acid observed in patients, patients with rhizomelic joints and connective tissue point of the first type and is observed both in the classic form of the disease, and in different variations. Values can vary from 200 to 900 $\mu\text{mol/L}$, to some extent depend on age. Now we discuss the cases of the appearance of excess phytanic acid in classical rhizomelic dot chondrodysplasia in newborns. In the laboratory of the authors involved in these cases was set to a normal level of phytanic acid in the plasma of patients under the age of one week (0.7 to 5.8 $\mu\text{mol/L}$). Patients aged from two to three weeks, have an increased level of phytanic acid from 9.1 to 13.2 $\mu\text{mol/L}$. As a rule, in patients at any age is impossible to determine the

level of plasmogen of red blood cells. In some cases there is a slight increase in the level of phytanic acid to a value of 15-35 $\mu\text{mol/L}$. Despite a detailed study of fibroblasts from several patients, explanation of this phenomenon still has not been found. The fact that the level of phytanic acid depends on the diet, can reduce the accumulation of phytanic acid due to diet. Patients with the Refsum's disease can achieve an almost normal level of phytanic acid in plasma by using a strict diet, accompanied by plasmapheresis, if necessary.

The authors of some articles have described cases of violation of the biogenesis of peroxisome, a deficit in D-bifunctional protein and acyl-CoA-oxidase in patients, analysis of plasma which had not revealed any deviations in the level of long chain fatty acids, phytanic, pristanic or bile acids. Obviously, peroxisome suspected violations should always be confirmed by the study of fibroblasts regardless of the results of the analysis plasma [6, 7].

CONCLUSION

Thus, the complex GC-MS analysis LCFA, pristanic acid and phytanic acid is an effective method to identify patients with peroxisome impairment, especially for diagnostics Zellweger syndrome spectrum, rhizomelic chondrodysplasia punctata type 1 and Refsum's disease. In disorders of biosynthesis of lipids, in particular with RCDP second and third types, and hyperoxaluria first type requires additional checking the content of other metabolites (level of plasmalogens in erythrocytes, glyoxylate, glycolate and oxalate levels in the urine). To diagnose diseases of peroxisome disorders recommended apply high-tech biochemical method GC-MS to determine the levels of very long chain fatty acids (VLCFA), phytanic acid and pristanic acid.

Therefore, for every laboratory is necessary to establish reference intervals for the performance of various markers of peroxisomal disorders of healthy children, that can't lead to significant errors in interpreting studies.

Competing interests

The authors declare that they have no competing interests.

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Characteristic of Shredded Made from Boiled Fish (*Euthynnus Affinis*) with Substitution of Okara

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ABSTRACT: Boiled fish is one of traditional fish process that people like due to the low price and high nutrient content. However, it has short shelf-life, salty taste and less appearance. Diversification product such as shredded is required to improve the quality of boiled fish. The purpose of this study was to improve the characteristics of shredded made from boiled fish with substitution of okara. Boiled fish was soaked at different water condition (cold and hot) and soaking time (10, 20, 30 and 40 minutes). The data indicated that hot water with soaking time 30 minutes showed higher decreasing salt contents compared with others. The resulting boiled fish was made with substitution of okara (0, 10, 20, 30, 40 and 50%). The results indicated that substitution 20% (w/w) okara showed better characteristic that others with water, protein, fat, ash content, TBA, soluble dietary fiber, insoluble dietary fiber values was 7.60%, 32.08%, 28.72%, 3.23%, 0.27 mg/kg, 1.39% and 7.67%, respectively. Moreover, sensory evaluation was proposed to measure the product's response by panelist and showed significant different in odor and texture characteristic.

Author Keywords: Boiled fish, Dietary fiber, Okara, Shredded

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INTRODUCTION

Fish production was abundant in Indonesia and it will be useless if just sold in raw fish or traditional process like boiled fish. Boiled fish is one of the traditional processed fish found in Indonesia. Little tuna (*Euthynnus affinis*) commonly used as boiled fish due to rich in protein and high amount of unsaturated fatty acid [1]. Boiled fish has great demand for Indonesian due to high nutrient content and more economical compared to red meat. However, it has some problem such as high salt content, harmful substances such as microbial and chemical contaminants and short shelf-life [2].

High salt consumption is a major factor in the increase in blood pressure, risk of stroke, kidney, obesity and stomach cancer [3]. Reduction salt content in boiled fish can be conducted by soaked in water. Less of information and processing technology cause low product diversification of fish. To improve the quality of boiled fish, one of the way was shredded. Shredded is chopped and dried by the addition of seasoning. Types of processed shredded fish is one of the diversified businesses processing of fishery products. Compared to other traditional forms of processing, shredded fish has relatively long shelf-life, which is still acceptable in storage for 50 days at room temperature. However, shredded has low fiber, so it needs to add some fiber to improve its quality. The addition of fiber also can reduce salt content and improve the texture properties. One of fiber which add to shredded was okara. Okara or known as tofu by product is classified in the industrial waste of agricultural products, can also be used directly as food product, easily damaged, and ends up as animal feed [4]. However, it has insoluble fiber 50.77%, 4.71% soluble fiber, protein 28.52%, 9.84% fat, 3.61% ash and 2.56% carbohydrates [5].

Due to okara showed higher protein and fiber, it is potential to add in foods and increase their characteristics. Boiled fish is low shelf-life and consumption, so combination between okara and boiled fish to be a shredded is required to improve the characteristic of shredded.

MATERIALS AND METHODS

The boiled fish with the length approximately 30 cm and weight 300 gram and okara was obtained from traditional market in Malang, East Java, Indonesia. Boiled fish was soaked in cold and hot water for 10, 20, 30 and 40 minutes, respectively. Subsequently, the resulting boiled fish (soaked in hot water for 30 minutes) was cooked with substitution of okara (w/w) 0%, 10%, 20%, 30%, 40% and 50%. Prior to cook, okara was steamed for 30 minutes and kept at room temperature. The shredded were mixed with 5% onions, 8% garlic, 2% coriander, 15%

brown sugar, 1% tamarind, 1% galangal, 50% coconut milk, lemongrass leave and stir-fried at 180 °C for 5 minutes. The formulation of shredded substituted by okara was resumed in Table 1. The water, protein, fat, ash contents, thiobarbituric acid (TBA), soluble dietary fiber, insoluble dietary fiber was determined according to [6] with slight modification. The sensory evaluation was conducted by panelists consist of 15 male and 15 female with ages between 20 to 50 years. They were selected from Fisheries and Marine Science Faculty and all had experience in sensory evaluation, not allergic to fish, consumption of boiled fish at least once per week and willingness to evaluate meat from boiled fish. Panelists were instructed to evaluate the color, salty taste of the products, odor, texture and hedonic test was performed. The experiments were conducted in triplicate and data were expressed as the mean \pm standard deviation. The data was analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). Differences between means were analyzed by analysis of variance (ANOVA) test with least significant different test at the resulting p -value lower than 0.05 ($P < 0.05$).

RESULTS AND DISCUSSION

We measured the salt contents of boiled fish and the data exhibited that soaked time for 30 minutes in hot water decreased the salt approximately $21.67 \pm 1.95\%$ were given in **Table 2** the differences of using water condition had effect for decreasing salt content. Decreasing of salt content in boiled fish due to hot water was capable to widen the meat's pores so the water entered and pulled the salt out from the meat faster. Although, soaked time 30 and 40 minutes did not showed significant different, soaked time 30 minutes selected due to time effectiveness. In addition, substitution of okara tends to decrease water and protein content but increase the fat content based on Table 3 the decreasing of water contents in shredded influenced by okara.

Okara had large pores and when it fried in hot oil, the water is easily removed and resulting low water content. On the other hand, during frying water loss as well as penetration of oil into food takes place [7]. Okara had low protein content, so when it substituted in shredded would decrease the protein content. Moreover, heat processing leads to reduction in protein value and total amino acid as described by [8]. Okara tends to increase the fat content due to the components of okara was capable to hold oil when pressed by spinner. Okara had high carbohydrate which indicated this component could absorb and hold oil in shredded. Moreover, the fat content influenced by water content due to water was removed and remained space which filled by the oil resulted increasing fat content. Ash content and TBA showed that there was no significant difference between the treatments. No significant different of TBA in treatments indicated that there was not oxidative damage in shredded. However, TBA could increase when storage days was longer and inappropriate storage condition would accelerate the lipid oxidation [9]. Hedonic test was conducted to measure the response of the product by panelist.

Table 1. Formulation of shredded substituted by okara

Formulation	Treatments of substitution okara (w/w)					
	0	10	20	30	40	50
Boiled fish	100	90	80	70	60	50
Okara	0	10	20	30	40	50
Onions	5	5	5	5	5	5
Garlic	8	8	8	8	8	8
Coriander	2	2	2	2	2	2
Galangal	1	1	1	1	1	1
Tamarind	1	1	1	1	1	1
Brown sugar	15	15	15	15	15	15
Coconut milk	50	50	50	50	50	50
Lemongrass leave	1	1	1	1	1	1

Table 2. Decreasing salt contents after treatment water condition and soaked time

Water condition	Treatments	Soaked Time (minutes)	Decreasing Salt Contents (%)
		10	1.74 \pm 0.97 ^a
Cold		20	10.25 \pm 2.99 ^b
		30	13.12 \pm 0.76 ^{bc}
		40	19.36 \pm 3.23 ^{cd}
		10	9.61 \pm 2.29 ^b
Hot		20	18.42 \pm 4.81 ^c
		30	21.67 \pm 1.95 ^d
		40	25.84 \pm 2.80 ^d

Table 3. Chemical characteristics of shredded substituted by okara

Substitution of okara (%)	Water (%)	Protein (%)	Fat (%)	Ash (%)	TBA (mg/kg)
0	10.63 ± 3.27 ^{ab}	36.91 ± 0.39 ^c	24.68 ± 1.26 ^a	3.85 ± 0.75 ^a	0.38 ± 0.27 ^a
10	12.81 ± 2.58 ^b	34.70 ± 1.05 ^{bc}	26.49 ± 3.36 ^{ab}	3.68 ± 0.50 ^a	0.24 ± 0.18 ^a
20	7.60 ± 1.48 ^{ab}	32.08 ± 1.72 ^b	28.72 ± 1.63 ^{abc}	3.23 ± 0.59 ^a	0.27 ± 0.24 ^a
30	7.22 ± 2.06 ^{ab}	28.72 ± 0.55 ^a	31.55 ± 3.17 ^{bcd}	3.36 ± 1.03 ^a	0.21 ± 0.15 ^a
40	8.31 ± 3.88 ^{ab}	25.81 ± 1.73 ^a	33.52 ± 1.52 ^{cd}	3.01 ± 0.85 ^a	0.51 ± 0.43 ^a
50	4.66 ± 1.45 ^a	26.87 ± 1.86 ^a	34.01 ± 1.98 ^d	2.66 ± 0.69 ^a	0.35 ± 0.31 ^a

Table 4. Sensory evaluations of shredded substituted by okara

Treatments of substitution okara (%)	Sensory evaluations			
	Color	Salty tastes	Odor	Texture
0	4.40 ± 0.48 ^{ab}	2.87 ± 0.12 ^a	3.58 ± 0.29 ^{ab}	4.07 ± 0.16 ^{bc}
10	4.05 ± 0.99 ^{ab}	2.97 ± 0.16 ^a	3.50 ± 0.20 ^{ab}	3.88 ± 0.16 ^{bc}
20	4.85 ± 0.87 ^{ab}	2.77 ± 0.04 ^a	3.90 ± 0.14 ^b	4.13 ± 0.17 ^c
30	3.37 ± 0.51 ^a	2.94 ± 0.28 ^a	3.45 ± 0.14 ^{ab}	3.57 ± 0.41 ^b
40	3.15 ± 0.77 ^a	2.55 ± 0.48 ^a	3.27 ± 3.45 ^a	3.02 ± 0.13 ^a
50	3.24 ± 1.00 ^a	2.30 ± 0.47 ^a	3.25 ± 0.30 ^a	2.88 ± 0.26 ^a

Shredded boiled fish with substitution of okara was served to panelist by random 6 alphabets (A to F) in one session in a taste panel including color, salty tastes, odor and texture using 7 point scale (1 is very dislike to 7 is very like). All of the sensory evaluation using p-value lower than 0.05 (*p<0.05)

Table 5. Nutrition composition of shredded with the best substitution of okara

Characteristics	Result
Water	7.60 ± 1.48
Protein	32.08 ± 1.72
Fat	28.72 ± 1.63
Ash	3.23 ± 0.59
Carbohydrate	26.90 ± 3.97
Salt	0.92
Total dietary fiber	9.06
- Water soluble	1.39
- water insoluble	7.67

According to Table 4 the result indicated that substitution of okara did not show significant different in color and salty tastes characteristic. However it showed significant different in odor and texture. Shredded fish with substitution okara is a relatively new product might be responsible for the insignificant difference in its acceptability. Basic tastes (sweet, sour, bitter, salt and umami) are one of the sensory evaluations of food, together with texture, pungency, aroma, appearance [10]. According to chemical characteristics and sensory evaluations, substitution 20% of okara or ratio boiled fish to okara was 80 to 20 was capable to increase the characteristic of shredded and nutrient composition can be seen in Table 5.

CONCLUSION

The soaked time 30 minutes in hot water can decrease the salt contents of boiled fish. Substitution of okara caused low water and protein content thus increase the fat content. The substitution of 20% okara improved the characteristic of shredded. Shredded can be considered as alternative way to increase the shelf-life of boiled fish. Nevertheless, variation of substitution and fish is necessary to improve the characteristic of shredded.

Competing interests

The authors declare that they have no competing interests.

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Efficacy of Endoscopic Interventions in Prevention of Gastroesophageal Bleeding in Patients with Liver Cirrhosis

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ABSTRACT: To analyze the survival of patients with liver cirrhosis, and to assess the effectiveness of endoscopic interventions in the prevention of portal genesis bleedings. To evaluate the efficacy of endoscopic interventions, our study included 449 liver cirrhotic patients with portal hypertension in the period from 1996 to 2015, admitted with bleeding from variceal bleedings or the threat of its recurrence. All patients were divided into 2 groups of the study. The main group included 239 patients treated between 2008 and 2015 in the control group - 210 patients in the period from 1996 to 2007. The analysis showed that the percentage of patients without recurrence of variceal bleeding when performing only endoscopic interventions was 27% (33 patients) in the control group and 54.2% (64) in the main group. With the phased tactics of portosystemic shunt performance after endoscopic interventions this figure amounted to 32.4% (45) and 109 (61.6%). In the structure of mortality of patients without cirrhosis in the long-term period (81 patients) with endoscopic interventions recurrence of bleeding were observed in 40.7% (33) cases in the control group and 68.1% (64 of 94) in the main group. In turn, when combined endoscopy and portosystemic shunting in the structure of the patients, without counting deaths from progressive liver cirrhosis, the proportion of absence of recurrence was 45.9% (in 45 of 98 patients) and 71.2% (in 102 out of 153 tracked in the remote period excluding deaths from cirrhosis). In the group of patients that do not carry out any endoscopic intervention and the patients received only conservative therapy only in 3 (10.7%) cases it was possible to avoid recurrence of bleeding, which determines the therapeutic ineffectiveness isolated attempts to reduce the risk of recurrence of hemorrhagic syndrome. Modern possibilities of endoscopic technologies have significantly improved the results of treatment and prevention of varicose bleeding or the threat of its recurrence, and the commitment to the phased tactics, with a combination of minimally invasive and traditional decompressive surgery, allowed to increase the survival rates of patients with 80% to 88% - up to 1 year and from 42% to 64% - to 3 years of follow-up.

Author Keywords: Liver Cirrhosis, Portal Hypertension Syndrome, Bleeding From Esophageal And Gastric Varices, Portosystemic Shunting, Endoscopic Ligation, Endoscopic Sclerotherapy.

INTRODUCTION

In modern hepatology chronic diffuse liver diseases are still relevant socio-epidemiological and clinical health problem [1, 2]. Bleeding from varicose veins of the esophagus and stomach (VVES) occurs in 80% of patients with liver cirrhosis (LC) with portal hypertension (PH) and is the most common and life-threatening complications [3, 4, 5]. The risk of its development during the first two years after the identification of the transformation of varicose veins of the esophagus is 30%, while the annual hemorrhagic syndrome develops in 12-15% of patients with VVES [6]. Mortality from the first bleeding ranges from 30 up to 60%, and within decompensated liver function reaches 76-100% [7,8].

The only radical treatment method is a liver transplantation, but in the absence of indications for transplantation, or because of impossibility of its performance within social and law issues, the main focus of the surgical treatment of these patients is the prevention of bleeding [9, 10].

Among the entire spectrum of surgical interventions, used in treatment and prevention of bleeding, minimally invasive technologies are of special interest. In this series, the most promising and competitive are the various techniques of endoscopic interventions on VVES. The development of these technologies brought them

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into the category of "first-line method", as in bleeding altitude so do in patients with high risk for its development [13, 14].

MATERIALS AND METHODS

The study included 449 patients with LC and PH admitted with bleeding from VVES or with the threat of its recurrence. Observation period was held from 1996 to 2015. All patients were divided into 2 groups of the study. The study group included 239 patients who received treatment between 2008 and 2015 in the control group - 210 patients which were observed between 1996 and 2007. In the main group endoscopic procedures have been used much more widely, both for prevention and for emergency indications. Endoscopic sclerotherapy (ES) was applied in 332 (73.9%) patients. Endoscopic ligation (EL) in 117 (26.1%) patients.

In the control group 102 patients were admitted in emergency order, and 119 - in the main group. Routinely hospitalized 108 and 120 patients, respectively. Age ranged from 19 to 66 years. The median age was 31.2 years. The men were 289 (64.3%), women - 160 (35.7%). During the emergency endoscopy esophageal varices was the source of bleeding in 81 (79.4%) patients in the control group and in 91 (76.5%) patients in the study group. Gastric cardia variceal bleeding (GC) was determined in 21 (20.6%) and 28 (23.5%) cases, respectively. In the early posthemorrhagic period 76 portosystemic shunt (PSS) were carried out in both groups, 19 - in the control group and 57 in the main. All patients had 1-2 sessions of endoscopic interventions with effective hemostasis and subsequent preoperative preparation to perform PSS in terms of 6 to 25 days after the bleeding.

The majority of patients had selective anastomosis performed - the distal splenorenal anastomosis (DSRA) - 49 (64.5%). Central anastomosis where performed in 27 (35.5%). In the group of central shunts performed splenosuprarenal anastomosis, laterolateral splenorenal, and H-shaped graft anastomosis with inserting of the internal jugular vein were performed.

RESULTS

In our observations, an effective endoscopic hemostasis was achieved in 76 (74.5%) patients in the control group, and in 84.9% (101 patients) in the study group, (Table. 1). In the GC bleeding group effective hemostasis elevated in 42.9% (9 of 21 patients) in the control group and 71.4% (20 of 28) in the main group. Operated 9 (42.9%) and 7 (25%) patients, respectively. Overall mortality was 7 (33.3%) and 4 (14.3%) cases.

In order to prevent bleeding after endoscopic hemostasis patients additionally had 1-3 sessions of EC or EL. The average re-ES sessions were carried out in 3-4 days. The progression of the various complications led to the transfer of 19 (18.6%) patients in the control group and 16 (13.4%) in the study group from functional class "B" to the class "C". The main reasons were growing of edematous-ascitic syndrome and progression of hepatocellular insufficiency. The class "C" progression of these complications observed in 14.7% (15 patients) and 6.7% (8), but it should be noted that by day 10-12 post-hemorrhagic rehabilitation from Class "C" in class "B" translated into 8 (7.8%) and 19 (16%) patients. In general, groups of improvement identified in 21.6% of patients in the control group and 33.6% in the study group, the deterioration to the 41.2% and 23.5% of cases, stable at 37.3% and 42.9% of patients.

In our observations Child-Pugh scale in the class "A" amounted to $6,2 \pm 0,1$ points, on a scale of MELD (Model for End-Stage Liver Disease) - $9,1 \pm 0,2$ points. In the class "B" - $8,4 \pm 0,3$ and $12,4 \pm 0,3$ points, respectively. In the class "C" - $11,6 \pm 0,3$ and $18,2 \pm 0,4$ points. Determination of the MELD was performed in 69 patients (from 2011), and was of fundamental importance in defining the terms for radical treatment. In our study, these figures reflect the condition of patients in period of development of hemorrhagic syndrome. Class "A" - 11 (15.9%) patients, class "B" - 36 (52.2%) and Class "C" - 22 (31.9%).

In 68.1% of cases the development of hemorrhagic syndrome in patients with LC is noted on the background of compensated and subcompensated disease with high short-term prognosis (3-month) survival ($P < 0,001$), defined on a scale of MELD, and corresponds to the class "A" Child -Pugh - 15,9% of patients with index $9,1 \pm 0,2$ points (MELD), class "B" - 52,2% ($12,4 \pm 0,3$ points). At maintaining the functionality reserve of the hepatocytes on the background of cirrhosis (MELD < 15 ; "A" and "B" Child-Pugh) leads to a favorable long-term prognosis without transplantation survival ($P < 0,001$), while the rate of mortality even at the first episode of bleeding in this group reaches 11,1%, followed by a multiple increase in this index at relapse.

Further the results of endoscopic interventions in the group of patients who were admitted in planned order were observed. In the control group 100% (108 cases) of interventions were carried out on the esophageal veins. In the main group in 6 of 120 (5%) cases routine vein ligation of the cardia of the stomach was performed. Bleeding after intervention noted in 6 (5.6%) of cases in the control group and in 2 (1.7%) in the study group.

Emergency surgery was required only in 2 (1.9%) and 1 (0.8%) patients. Mortality in the control group was 0.9%, and in the there was no lethal outcome in the main group.

Summing up the results of all interventions following can be noted. Endoscopic procedures efficiency was 82.4% (173 of 210 patients) in control group and 90% (215 of 239) in the main group. Emergency operation after endoscopic manipulation was performed in 23 (11%) and 15 (6.3%) patients. Overall mortality was 10.5% (22 patients) and 5% (12), respectively (Fig. 1).

Table 1. The efficacy of endoscopic hemostasis and mortality rate, depending on the Child- Pugh functional class

Index	Control				Main			
	Functional class			Total	Functional class			Total
	«A»	«B»	«C»		«A»	«B»	«C»	
Total amount	18	48	36	102	19	55	45	119
	17.6%	47.1%	35.3%	100%	16.0%	46.2%	37.8%	100%
Effective hemostasis	16	38	22	76	18	47	36	101
	88.9%	79.2%	61.1%	74.5%	94.7%	85.5%	80.0%	84.9%
Ineffective endoscopic hemostasis, or inability of intervention	2	10	14	26	1	8	9	18
	11.1%	20.8%	38.9%	25.5%	5.3%	14.5%	20.0%	15.1%
Mortality	0	4	10	14	0	3	6	9
	0.0%	8.3%	27.8%	13.7%	0.0%	5.5%	13.3%	7.6%
Dissection surgery's	2	10	9	21	1	8	5	14
	11.1%	20.8%	25.0%	20.6%	5.3%	14.5%	11.1%	11.8%
Mortality (after surgery)	0	2	5	7	0	1	2	3
	0.0%	20.0%	55.6%	33.3%	0.0%	12.5%	40.0%	21.4%
General mortality	0	6	15	21	0	4	8	12
	0.0%	12.5%	41.7%	20.6%	0.0%	7.3%	17.8%	10.1%

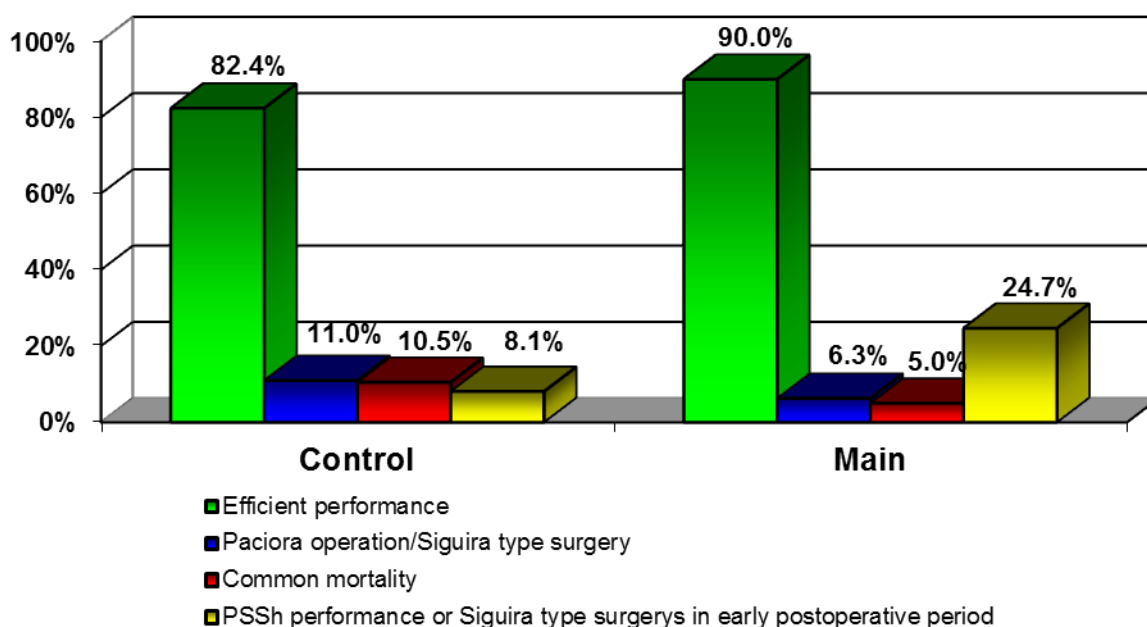


Figure 1. Short-term results of endoscopic interventions

It should be noted that in the study group good hemostatic effect and tactics undertaken by post-hemorrhagic rehabilitation allowed to expand a greater degree opportunities for the use of combined-stage tactics. After only three weeks after the hemorrhage episode with an effective endoscopic hemostasis performed PSS was performed in 17 (8.1%) patients in the control and 59 (24.7%) in the study group.

In the late period 319 patients were observed in both groups. Also, from the analysis of long-term results observation patients who were on a background of ineffective endoscopic hemostasis were performed various options for emergency uncoupling operations. In general 72 patients were excluded from the study.

The main parameter for evaluating long-term results was the analysis of the haemorrhage recurrence. In view of decompressive effect of performed PSS in some patients, they were not included in to the study. Within one month after discharge 10 (8.2%) of 122 patients from the main group and 5 (4.2%) patients in of 118 in the study group were admitted to different clinics (Fig. 2).

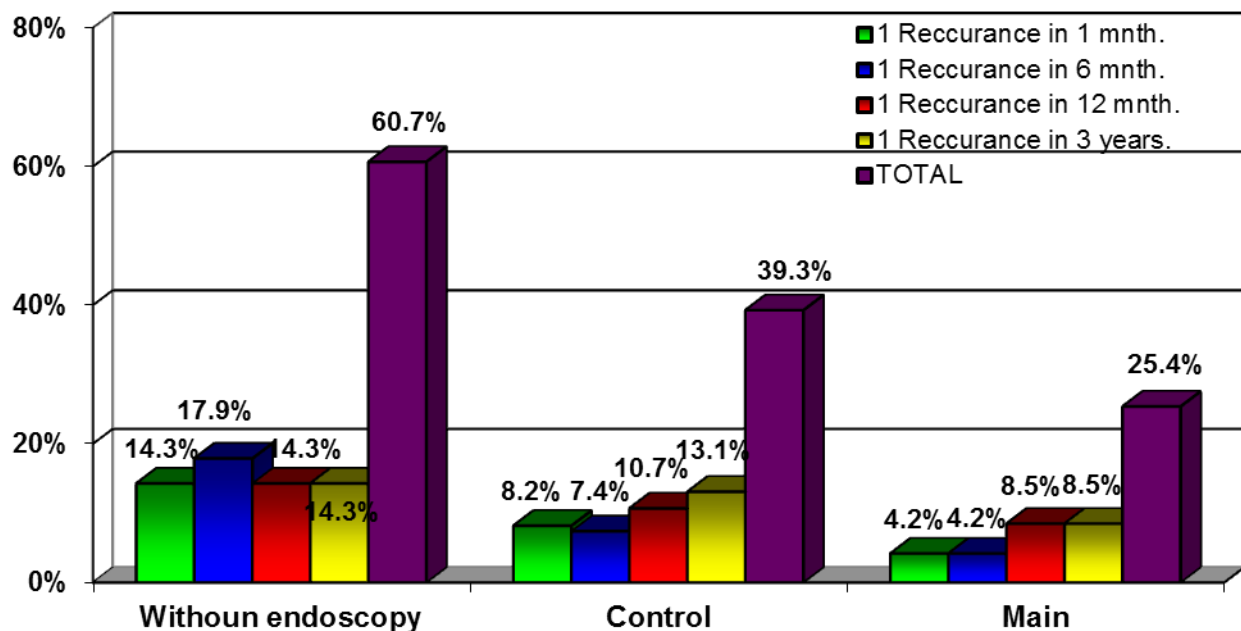


Figure 2. Frequency of rebleeding after endoscopic interventions

Analysis of patients with conservative, including a Blackmore-Segstaken tube hemostasis is of a special interest. Of these 28 patients, bleeding in the three-year period of observation developed in 17 (60.7%), including 13 (46.4%) in the periods up to a year after the initial episode.

Due to mortality in the remote period, patients were divided into those with LC progression and ones with recurrence of hemorrhagic syndrome. In the various periods of follow up because of increasing liver failure died 5 (4.1%) 15 (12.3%) in the control group and 3 (2.5%) 10 (8.5%) in the study group. Bleeding from 4 (3.3%) to 9 (7.4%) and 2 (1.7%) to 4 (3.4%), respectively. In general, in control group in the remote period of up to 3 years, 41 (33.6%) patients died due to increasing liver failure and 22 (18%) patients from hemorrhage. In the main group, the figures were lower and amounted to 24 (20.3%) and 1 (9.3%) patients, respectively. Mortality among patients with bleeding was 45.8% in the control group (22 deaths out of 48 patients) and 36.7% in the study group (11 out of 30 died).

The analysis showed that: the proportion of patients with effective hemostasis within three years without recurrence only with performance of endoscopic procedures was 27% (33 patients) in the control group and 54.2% (64 patients) in the study group. In the phased tactics with the performance of PSS after endoscopic interventions mentioned rates were: in the main group - 61.6% (109 patients) In the structure "no mortality" in patients with LC in the remote period (81 patients) with endoscopic interventions there was no recurrence of bleeding observed in 40.7% (33) cases in the control group and 68.1% (64 of 94) in the main group. In turn, in patients with combined endoscopy plus PSS, not including those who died from progressive LC, absence of bleeding recurrence was 45.9% (in 45 of 98 patients) and 71.2% (102 out of 153 tracked at a remote period, excluding died from LC complications). In the group of patients that did not underwent any endoscopic procedures, and were receiving only conservative therapy only in 3 (10.7%) cases it was possible to avoid recurrence, this determines the therapeutic ineffectiveness of isolated attempts in the risk of recurrence reduce.

DISCUSSION

Stage care in the tactics of management of patients with LC with the threat of hemorrhagic syndrome encouraged by many hepatological schools. In countries with developed transplant service minimally invasive intervention, such as endoscopic or endovascular manipulation, can extend the waiting period for a liver transplantation, as

mortality from hemorrhagic syndrome on the waiting list exceeds 25%. For countries with a lack of opportunity of radical treatment, the risk of rebleeding dictates necessity of optimization of therapeutic and preventive measures without considering the probability of liver transplantation in the future. Studies have shown that the modern arsenal of endoscopic technologies ensures adequate hemostasis. But to improve the long-term results over a third of patients required to take additional preventive measures, as even a phased implementation ligation or sclerotherapy does not achieve complete eradication of veins. Against this background and different technical capabilities, combining preventive methods are used. In particular, endoscopic procedures are used as a means of influencing directly on the source of the bleeding, but decompressive effect is achieved by performance of different portocaval bypass surgeries. These operations are divided into two groups: traditional and endovascular. The essence of transjugular intrahepatic shunting (Transjugular intrahepatic portosystemic shunting - TIPS) is in the combination of intrahepatic branches of the portal vein to the hepatic vein. Alternatively PSS, and TIPS can also be attributed to as partial, due to the possibility of creating different shunt diameters. In our view, PSS should still continue to be considered as an option method of portal decompression, especially in patients with ineffective pharmacologic and endoscopic treatment, and in the absence of indications for liver transplantation. The use of different options of endoscopic interventions, even with the dynamic control and with the use of additional ligation sessions or sclerotherapy, allowed to achieve 78% annual survival rate. In the group of patients with PSS mentioned rates were: 88%. Survival at 24 months was only 54% in the endoscopy group, while the combined tactics improved this index up to 78%. In terms to 3 years of observation, the figures were 34% versus 64% ($P < 0,001$).

In general, PSS should be carried out only by certain indications. For functional class "C" patients endoscopy remains a priority method of hemostasis and bleeding prevention. Assuming stabilization of patients state and transfer to functional classes A and B, the question of decompressive operations will be opened. In other cases it is advisable to use only minimally invasive techniques. In particular, endoscopic and endovascular interventions, since this group of patients due to severity of LC, are already regarded as potential candidates for radical treatment.

CONCLUSIONS

In 68.1% of cases hemorrhage development in cirrhotic patients is observed on the background of compensated and subcompensated course of the disease with a high forecast short-term (3-month) survival ($P < 0,001$). Mentioned is defined by MELD scale. Class "A" on the Child-Pugh - corresponds to 15.9% of patients with index $9,1 \pm 0,2$ points, class "B" - 52,2% ($12,4 \pm 0,3$ points).

Maintained hepatocytes functional reserve on the background of cirrhosis (MELD < 15 points and classes "A" and "B" on the Child-Pugh) leads to a favorable long-term prognosis of "without transplantation" survival ($P < 0,001$), while the mortality rate on the first episode of bleeding in this group of patients reaches 11.1%, followed by a multiple increase in this index at relapse.

Prognostic value of prophylactic endoscopic interventions is determined by the reduction of bleeding recurrence. So, using only conservative prophylactic methods in a period of up to 3 years of observation, complication recur in 60.7% of patients with post-hemorrhagic mortality indicator - 46.4%, while their implementation reduces these values to 18.0%, respectively.

Modern opportunities of endoscopic technologies have significantly improved the results of treatment and prevention of varicose bleeding or the threat of its recurrence. In this landmark of commitment tactics, with a combination of traditional and minimally invasive decompressive surgery, allowed to increase the survival rates of patients from 80% to 88% - up to 1 year and 42% to 64% - to 3 years of follow-up.

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Competing interests

The authors declare that they have no competing interests.

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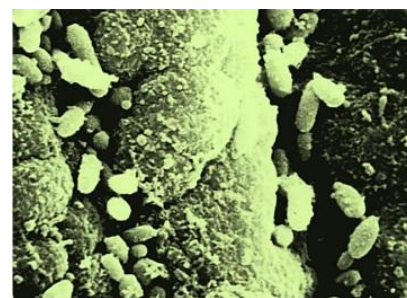
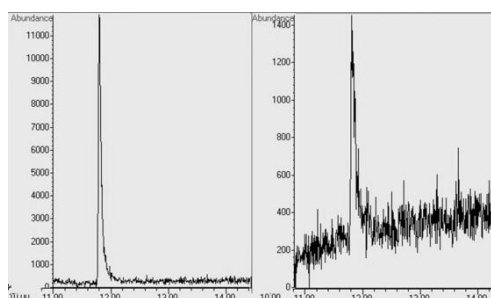
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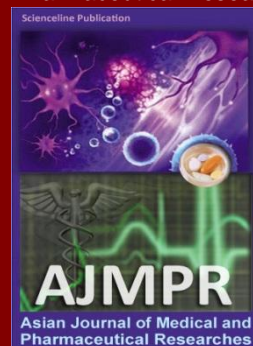
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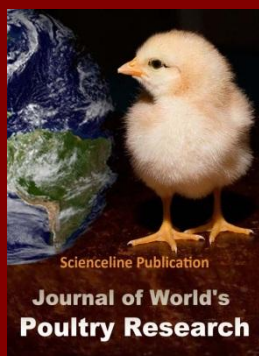
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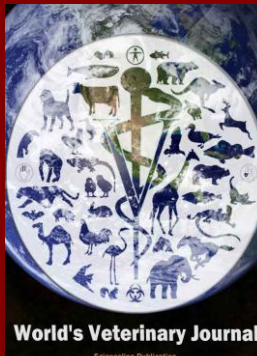
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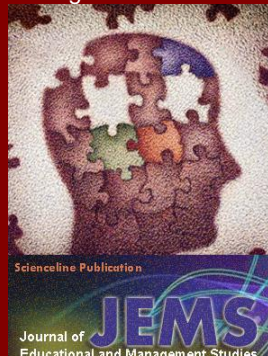
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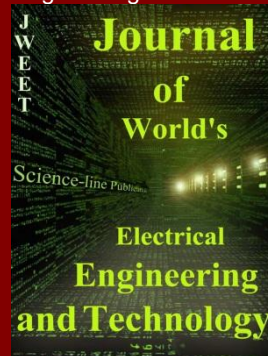
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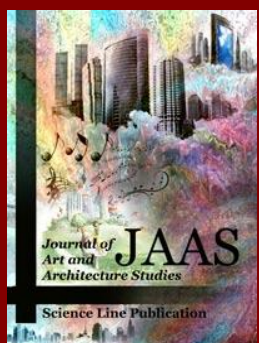
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