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Comment on Optical Methods in Blood Studies Upon Evaluation of Severity Rate of Diffuse Liver Pathology

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Abstract— **Paper Increase in efficacy rate of early diagnosis of fibrotic changes in the liver remains to be the burning issue in modern hepatology as it is difficult to diagnose the early stage of fibrosis due to its asymptomatic course. Progression of fibrosis in 60-80% of cases results in diagnosis of the disease at the stage of liver cirrhosis. This results in the increased rates of hospitalization, invalidity and mortality as well as economic losses due to therapy of liver cirrhosis and its complications including liver transplantation. The degree of fibrosis and its progression rate predetermine prognosis of the disease and the choice of therapy. Specification of the stage of the disease in hepatologic diagnosis is an obligatory component along with etiology and activity rate of the process. Therefore, it is of importance to diagnose the disease at its early stages and to evaluate the dynamics in accumulation of the fibrotic tissue.**

Index Terms—**dielectrophoresis, diffuse liver pathology, erythrocytes, optical methods.**

I. Introduction

Measurement of fibrosis not only helps to stage the severity of disease, it allows serial determination of disease progression. The level of fibrosis may play an important role in clinical management and determine patients' prognosis. For example, aggressive therapy is more appropriate in HCV-infected patients with advanced fibrosis. Further, the fibrosis progression rate is an important predictor of the time to develop cirrhosis [1].

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It is essential to measure fibrosis accurately, given the growing prospect of antifibrotic therapies and the need to track their efficacy. Moreover, with growing evidence that fibrosis is reversible, methods will need to assess both progression and regression accurately. For example, specific therapy leads to a reduction in fibrosis in a number of diseases, including alcoholic, autoimmune liver disease, hepatitis C, hepatitis B, and others [2-5].

Percutaneous liver biopsy has traditionally been considered to be the gold standard test to assess liver fibrosis. However, a variety of non-invasive tests have been advanced as potential alternatives to biopsy. These include clinical signs, routine laboratory tests, quantitative assays of liver function, markers of extracellular matrix synthesis and/or degradation, and radiologic imaging studies. In addition to individual indicators of fibrosis, combination tests, and a number of models for predicting liver fibrosis have been developed.

The ideal method to measure fibrosis would be simple, noninvasive, reproducible, inexpensive, accurate, and readily available. Unfortunately, none of the currently available approaches fulfills all of these criteria.

In our study [6], it was proposed to use the optical methods in studies of erythrocytes and blood serum in diagnosis of patients with diffuse liver disease with various degree of fibrosis. The findings of previous studies of electrical and visco-elastic parameters of erythrocytes, ellipsometric indices of blood serum in patients with diffuse liver disease enabled to obtain the encouraging pilot results concerning successful diagnosis of the stage of the disease [7-9].

The optical methods applied in evaluation of formed elements and blood serum are known to have some advantages that are significant for studies of biological objects. First of all, their high sensitivity rate when measuring low concentrations of bioorganic compounds in solutions or when applying these solutions to hard substrates is very crucial. Second of all, they do not destroy biological objects, numerous of which being of a complicated structure. Third of all, these methods enjoy high operational efficiency of measurements which do not require any special conditions (i.e. high vacuum, heating or cooling of a sample under study, special ionizing radiation, and the use of fluorescent tags).

The method of dielectrophoresis (DEP) was applied to study electrical and visco-elastic parameters of erythrocytes in patients with various degree of fibrosis. The essence of the

method lies in the fact that individual interaction of each separate suspension cell with the non-uniform alternating electric field (NUAEF) is accompanied by polarization of its positive and negative charges (Fig. 1, left part).

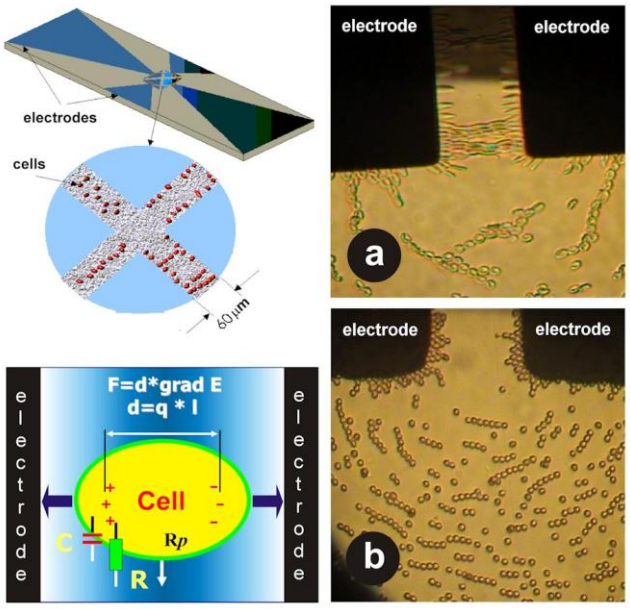

Fig. 1. Left part: Schemes of DEP: cell (up) and process (down). Right part: Division and deformation of erythrocytes influenced by non-uniform alternating electric field at frequency of 1 MHz: (a) High deformation amplitude in control group (F0); (b) Low deformation amplitude in patients with severe liver fibrosis (F3-F4).

Such polarization results in appearance of the induced dipole moment in the cellular volume. The dipole moment and the polarization coefficient of a single cell are connected with the spatial arrangement and movement of electric charges within the whole cellular volume. Amplitude-and-frequency-related characteristics of a cell in NUAEF serve as a good indicator of physical-and-chemical properties of its membrane and cytoplasm as well as depict the condition of its biological activity. Changes in electrical, visco-elastic parameters of erythrocytes reflect pathological processes including the ones in cases of the presence of diffuse liver diseases and progression of fibrosis [9].

Previous analytical studies [10-12] proved that both internal and external structures of a cell could be selectively studied by means of changes in frequency of the external generator. At lower frequencies ($5\square10^4$ Hz, 10^5 Hz) they could study electrical characteristics of the membrane and cellular surface while at high frequencies ($5\square10^5$ Hz, 10^6 Hz) it was possible to study electrical parameters of its cytoplasm.

All plasmatic membranes, in spite of having some structural-and-functional peculiarities, are known to have the same composition pattern [13]. Schrier S. L. [14] proved the ability to use structural-and-functional characteristics of the erythrocytic membrane as a model for studies of other membranes which cannot be studied directly, e.g. the ones of hepatocytes. Thus, an erythrocytic membrane can serve as a sort of "mirror" reflecting the conditions of other membranes.

An erythrocyte is a biological substance which secures transport of oxygen and participates in support of homeostasis in organs and tissues of the human body. Due to a large number of erythrocytes present in the body the key physical-and-chemical properties of blood are determined predominantly by these cells. As there is a close connection between the disturbances in liver functions and blood homeostasis, the increase in degree of fibrosis in cases of diffuse liver diseases is to cause changes in characteristics of erythrocytes as well [15].

On the other hand, shifts in electrical and visco-elastic properties of erythrocytes tend to aggravate the course of diffuse liver diseases and lead to disturbances in microcirculation and tissue hypoxia which, in its turn, is known to stimulate fibrogenesis (Fig. 1, right part) [16].

Application of blood cells in evaluation of the degree of liver fibrosis is not an innovative technique. There exist well-known non-invasive tests bases on indirect markers of fibrosis which comprise calculation of thrombocytes' count (i.e. APRI test, Model 3, FIB-4, FibroIndex, Forns' index etc.). However, it is worth mentioning that the decrease in the level of thrombocytes can be observed already at the advanced stages of fibrosis while this index is less informative at the early stages of the disease [17].

Changes in parameters of red blood cells, components of blood serum with further shifts in their optic characteristics are known to be registered at the initial stages of fibrogenesis. This adds to the prognostic value of the negative results in application of optic methods (66.7%), i.e. diagnosis of "light" fibrosis. These data are comparable with the results of application of the method of indirect elastometry, i.e. transient elastography or FibroScan (Echosens, Paris, France) aimed to diagnose the early stages of fibrosis [18].

Ability to define the degree of fibrosis in cases of diffuse liver pathology of any etiology serves as an advantage of the above described optic methods. The number of well-known non-invasive tests (i.e. ActiTest, NashTest, AshTest, SteatoTest) enable to evaluate the degree of severity of fibrosis connected with chronic viral B and C hepatitis, non-alcoholic fatty liver disease and steatosis, correspondingly. This foresees the necessity of preliminary evaluation of the genesis of the disease as the accuracy of diagnosis of the degree of fibrosis depends upon it [18].

This study comprised the patients with viral, alcoholic and mixed genesis of the disease with various degree of fibrosis. The degree of fibrosis could be easily detected using the above-described optic methods in cases of non-alcoholic fatty liver disease, drug-induced liver pathology, autoimmune hepatitis and cumulative diseases. This is due to the fact that changes in various parameters of electrical, visco-elastic properties of erythrocytes, optic characteristics of blood serum take place. The complexes of parameters that are significantly important for this or that degree of fibrosis in cases of diffuse liver pathology of various etiology can be obviously different. But all of them get analyzed by means of special algorithm upon verification of the liver fibrosis.

The above described method has been patented as "The method of differential diagnosis of liver diseases" (2004) [19], and "A non-invasive method of diagnosis of the degree of liver fibrosis" (2015) [20].

At the Siberian Scientific Research Institute for Metrology,

ROSSTANDART, Novosibirsk, Russia the conduct studies of electro-optical system of evaluation of cells by means of dielectrophoresis, develop state standards of physical units reflecting electrical and visco-elastic parameters of erythrocytes. .

Thus, evaluation of the degree of liver fibrosis (a stage of the disease) by means of studies of electrical and visco-elastic properties of erythrocytes enables to define the prognosis of the disease no matter what etiology of the process can be. This approach secures prevention of serious complications in the patients' conditions upon taking study samples and increases physical and psychological tolerance of the patients. Treatment of the obtained results with the help of special computer software increases objectivity of evaluations of the findings under evaluation no matter what qualification and experience of a specialist in charge of this study is. Diagnosis of the degree of liver fibrosis at early stages of the disease demonstrates the value of this method if applied at the pre-cirrhotic stage of the disease. Low cost and labor input (it takes 3-5 minutes to conduct the test) precondition increase of its availability in diagnosis of the degree of liver fibrosis on the level of mass diagnostics including the screening studies as well.

Besides the dielectrophoresis technique we also applied in this study the methods of ellipsometry, IR-spectroscopy and Raman spectroscopy of blood serum of the same patients under study which enables us to increase the levels of specificity and sensitivity for diagnosis of the degree of liver fibrosis.

Along with aggravation of the liver disease more pronounced changes in blood serum occur. They are due to dysfunction of hepatic protein synthesizing function, aggravation of syndromes of cytolysis, cholestasis, and the immune inflammatory one. Similar disturbances cannot but affect the optical characteristics of blood serum that was demonstrated in our previous pilot studies [7].

The ellipsometric technique is based on the analysis of the condition of polarization of the light beam, reflected from the surface under study. Ellipsometry possess an extremely high sensitivity to the presence on the surface under study of any layers with any thickness including the ones with submonolayer surfaces [21]. The spectral ellipsometry enables to define the n(λ) dispersion characteristics and the total thickness of the films produced on the basis of blood serum. Besides, the reflecting ellipsometry enables to define the degree of evenness of coverage of the sample with the biochemical material which is important in cases of evaluation of the composition of blood serum determining its viscosity rate.

Evaluation of ellipsometric indices of thin film findings obtained from blood serum of patients with various degrees of severity of liver fibrosis enabled to reveal significant increase of retraction index in combination with decrease of film thickness along with fibrosis increase.

In cases of severe fibrosis similar changes can be attributed to reduction of synthesis of the number of compounds in the liver (i.e. blood serum protein, coagulation factors, cholesterol, triglycerides and others) and they become crucial for the film thickness. Prevailance of nonhomogeneous films in cases of severe fibrosis ("Microscan", ISP SB RAS, Novosibirsk, Russia - high resolution scanning ellipsometer) can be obviously attributed to the degree of biochemical disbalance in blood serum induced by liver cirrhosis [22]. The index of refraction is to a great degree determined by the components of cytolytic syndromes (ALT, AST) and the ones of cholestasis (total bilirubin , GGT); the degree of intensity of the components tends to increase in severe fibrosis (Fig. 2) [16].

Such changes were determined based on significant dislocations on the IR-spectra, where the peak areas and intensity determining the secondary structure of proteins are registered. Special attention was paid to evaluation of the Amide I, Amide II, Amide III amide groups which are connected with absorption of IR-radiation by the O=C-N-H fragments, the latter being sensible to the secondary structure of protein molecules (α-helix, β-sheet, random coil). Changes of the secondary structure of protein molecules, in their turn, are judged by the presence of pathological process in the organism, including the diffuse liver pathology with various degree of fibrosis (Fig. 3).

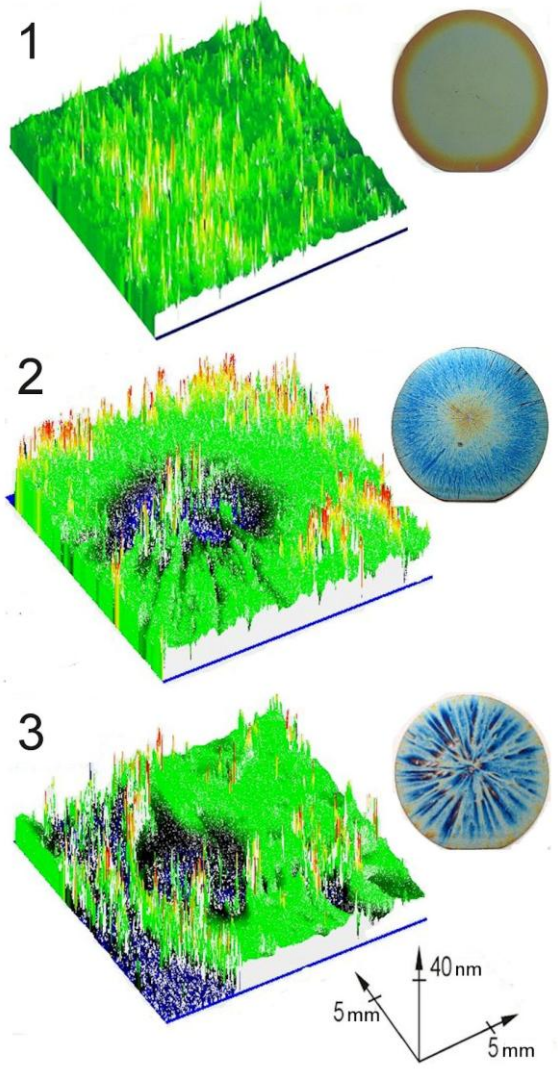

Fig. 2. Thickness distribution of three-dimensional thin films on the surface of plates processed from blood serum of control F0 (1), patients with light F1-F2 liver fibrosis (2) and severe F3-F4 liver fibrosis (3).

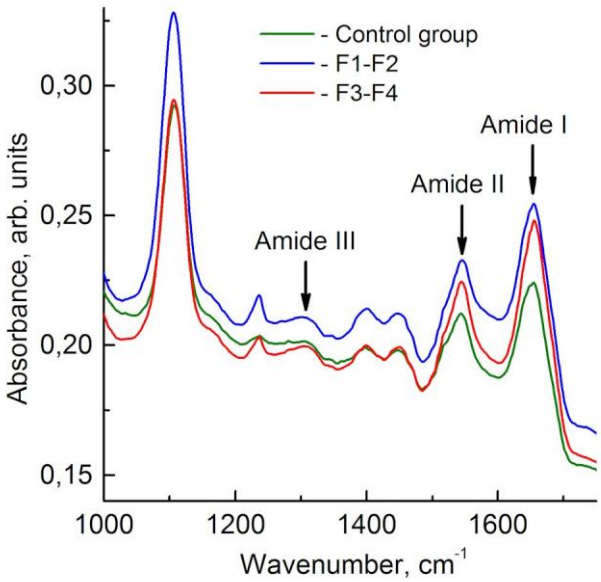

Fig. 3. Fragments of IR-spectra of blood serums in control group (F0), patients with light F1-F2 liver fibrosis and severe F3-F4 liver fibrosis.

Raman-spectroscopy serves as a powerful method of study of chemical structure of liquid samples of blood serums. The presence and relative intensity of characteristic peaks in the Raman-spectrums can speak in favor of the presence of changes in the chemical structure of the albumin molecules of blood serum which, in its turn, can confirm the presence of characteristic pathological processes in the human organism.

The diagnosed decrease in intensity rate of signals at 1005, 1157 and 1520 cm^{-1} in the Raman-spectrum at the background of aggravation of liver fibrosis can be obviously connected with the significant shifts in metabolism of carotins, their transformation in the A vitamin that is closely connected with changes in the hepatic function (Fig. 4) [16].

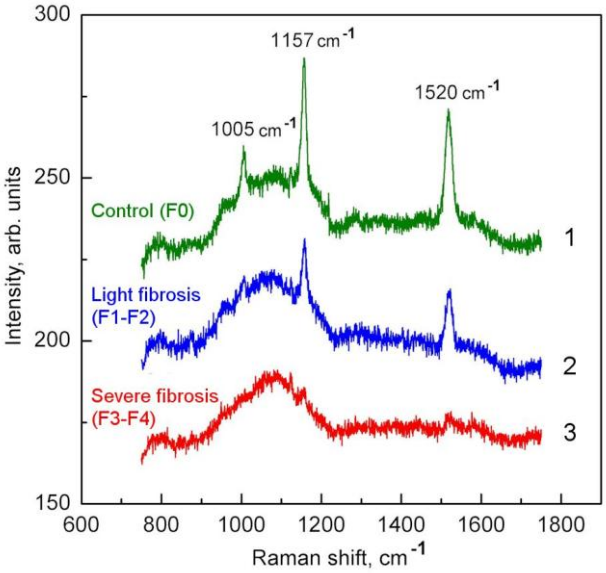

Fig. 4. Raman spectra of the samples of liquid blood serum in control group F0 (1), patients with light F1-F2 liver fibrosis (2) and severe F3-F4 liver fibrosis (3).

The attempt of further combined application of optical differences in parameters of erythrocytes and blood serum used to determine the degree of fibrosis is based on the well known facts about strong interrelation between red blood cells and blood serum components. There is a constant interchange of lipid components between them. Viscosity level and erythrocytes aggregation at diffuse liver disease are known to be influenced to a definite degree by thrombocyte aggregation, hypercholesterolemia, hypertriglyceridemia, fatty acids, and ionic composition. Increase in blood viscosity upon hyperlipidemia is predetermined by the capacity of high density lipoproteids to affect the functional state of regular blood elements, to reduce their electro-kinetic potential and as the result to increase the aggregation capacity and rigidity of membranes.

For example, one can observe inhibition of anticoagulating activity with strong depression of fibrinolysis and activation of blood coagulation service, degradation of rheological blood indexes [23, 15] under the influence of alimentary hyperlipidemia. T. Y. Leonova and co-authors [24] have determined that erythrocytes can absorb on their surface lipoproteids of blood plasma charged by cholesterol (in the form of VLLD, LHD and LLD). This capability is attributed to the presence of lipoproteids in blood serum and the need of erythrocytes in cholesterol. Constant cholesterol metabolism is known to happen between erythrocytes and lipoproteids adsorbed on their surface. The most intensive metabolism of cholesterol is observed between red blood cells and LHD. In this case a blood serum enzyme, i. e. lecithin-cholesterol-acyltransferase (LCAT) is known to play a significant role. Its synthesis takes place in the liver and is to a great extent predetermined by its state. One can conclude that diffuse liver pathology is accompanied by decrease of LCAT level with further increase of free cholesterol in blood serum and later on in erythrocytes. This is secured by cholesterol reducing capacity of erythrocytes that, on the one hand, influences upon the intensity rate of cholesterol metabolism between the lipoproteids of blood serum and blood cells, and, on the other hand, it supports a physiological level of cholesterol in erythrocytes. Disturbances in this biologically important mechanism of cholesterol metabolism can result in hypercholeterolestremia, the latter being an important risk factor in development of atherosclerosis, IHD and a potential cause of pathologic condition of optical parameters of blood serum [25].

It has been established that large-molecule proteins of blood serum tend to settle on erythrocyte membranes. This results in "screening" of the charge of erythrocytes as well as reduction of its dipole moment and the surface negative charge. Streiff et al. established [26] that zeta potential of erythrocytes correlated with changes in concentration of protein fractions. Stoltz et al., in their turn, revealed that the globulin fraction favored rapid decrease of electrophoretic mobility of erythrocytes while the albumin one would practically not influence upon their mobility in the electrical field. Other researchers [23, 27] proved that changes in the electrical charge would mainly depend upon the ratio of protein fractions, i.e. the greater the shift from the normal physsiological state is, the lower the electrical charge

becomes. The decrease of electrical charge of erythrocytes and increase of blood serum globulin level are attributed to the absorption of globulin molecules on the cells surface, the latter traditionally possessing the lower electrical charge. The idea that globulins can be adsorbed on the surface of erythrocytes irrespective of their antigenic structure has been confirmed by numerous researchers, including the results of Pirofsky B., Cordowa M., Imel T. (1962). Dysprotoneimia associated with the increased γ-globulins has been observed in cases of diffuse liver disease (DLD) upon the pronounced immuno-inflammatory syndrome [16]. It can be assumed that decrease of electrical charge of erythrocytes in patients with DLD can be predetermined by changes in ratio of protein fractions.

On the other hand, it has been established that in cases of diffuse liver damages increase of high-molecular weight protein (fibrinogen) in blood plasma is observed, which reduces the negative charge of erythrocyte and hampers their approach [28]. According to V. A. Levtova and the co-authors [29], the fibrogen molecules serve as bridges connecting the cells. Adsorption on the surface of erythrocytes of plasma proteins (fibrin, fibrogen) tends to favor aggregation and adhesion of erythrocytes. It can serve as one of the reasons of the increase of erythrocytes aggregation index in patients with DLD revealed in this study upon examination by method of dielectrophoresis.

In our previous study [7] we have established the correlation of the optical indices of blood serum, intensity of IR-spectra resonances with lipid and protein components. Consequently, the relations discovered between electrical and visco-elastic parameters of erythrocytes and the optical indices of blood serum seem to be logic.

The results of comperative evaluation of the set of optical methods for assessment of erythrocytes and blood serum in patients with diffuse liver pathology aimed to diagnose the degree of fibrosis and the results of liver biopsy (a "golden standard" for estimation of the degree of fibrosis) demonstrate sufficiently high values of sensibility (78.6%) and specificity (87.7%), prognostic value of positive (91.7%) and negative result (66.7%) as well as the index of accuracy (81%) [30]. The increased levels of these values as compared to the isolated study of erythrocytes enable to assume the necessity of application of the “set of optical methods” for blood studies in cases of non-invasive diagnosis of the degree of liver fibrosis. The most important are the perspectives of diagnosis of the early stages of liver fibrosis (according to the data of prognostic value of the “-“result).

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