2013 © Curr. Issues Pharm. Med. Sci. Vol. 26, No. 1, Pages 109-113

Current Issues in Pharmacy and Medical Sciences

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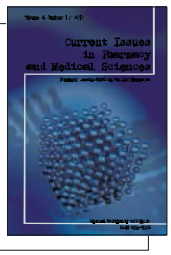

Diagnosis of experimental stetohepatosis using ultrasound shear wave elastography

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ABSTRACT

According to available literature data, NAFLD may play crucial role in the pathogenesis of type 2 diabetes and other conditions connected with insulin resistance. In order to study the essence of the NAFLD, we have created an experimental model of the steatohepatosis. The mixed diet for 8 weeks consisting of standard food (47%), sweetened condensed milk (44%), vegetable oil (8%) and vegetable starch (1%) develops non-alcoholic steatohepatosis in the animals undergoing the experiment. The morphological signs of the non-alcoholic steatohepatosis comprised as follows in the hepatocytes of the rats undergoing the experiment: presence of fine-drop fattiness (fine-drop steatosis) and accumulation of fat vacuoles shifting the nucleus towards the cell peripheral. Substantial increase in the liver pulp of the animals undergoing the experiment defined using the ultrasound shear wave elastography technique is indicative of the presence of the non-alcoholic steatohepatosis. The ultrasound shear wave elastography technique can be used as a non-invasive diagnosis marker of the non-alcoholic steatohepatosis. The said diagnosis technique has been recommended for the first time.

Keywords: steatohepatosis, non-alcoholic fatty liver disease, ultrasound liver elastography

INTRODUCTION

For the recent years, many researches have focused on a non-alcoholic fatty liver disease (NAFLD) as a multifactor and non-specific affection in obese Type 2 diabetic patients and against a dyslipidemia. [1,3,15].

Insulin resistance, hypertriglyceridemia, oxidative stress, and inflammatory and destructive changes in the liver underlie the formation of a fatty hepatosis [12,14]. It has been proved that the NAFLD increases the risk of cardiovascular complications and a metabolic syndrome. The fact has been proved by the decrease of adiponectin concentration, the last having an antiatherogenic effect [2]. Furthermore, an expressed endothelial dysfunction is observed in the NAFLD patients being a sub-clinical feature of atherosclerosis [7]. The NAFLD develops gradually and manifests itself in steatosis, steatohepatitis and fibrosis stages.

In order to study the essence of the NAFLD, its role in the development of metabolic syndrome, Type 2 diabetes and cardiovascular diseases and the effect of drugs on the NAFLD, we have created an experimental model of the steatohepatosis.

MATERIALS AND METHODS

All experiments were carried out according to the National Institute of Health Guidelines for the care and use of laboratory animals and the European Council Directive on 24 November 1986 for Care and Use of Laboratory Animals (86/609/EEC), and approved by the Local Ethics Committee. The experimental model of the non-alcoholic steatohepatosis has been reproduced in mature rats of 180-200g weight that were kept in a vivarium under standard keeping conditions and sanitary and hygienic conditions. The experimental animals were divided into 2 groups. The first control group consisted of 8 rats, and the

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DOI: 10.12923/j.2084-980X/26.1/ a.24

second experimental group suffering from the non-alcoholic steatohepatosis consisted of 12 animals.

The rats were fed standard food – «Purina rodent chow» (fat – 20.6%, protein – 32.4%, and carbohydrate - 47%) and water *ad libitum* for the first two weeks. Following which the animals were randomized by two groups. The animals belonging to the first control group were feed standard food and water *ad libitum* for 7 weeks. The animals belonging to the experimental group were on a mixed diet for 7 weeks consisting of standard food (47%), sweetened condensed milk (44%), vegetable oil (8%) and vegetable starch (1%) (fat – 29.6%, protein – 14.8%, and carbohydrate – 55.6%) (diet No 3 11024. Research Diets, New Brunswick. NJ (West D., et al., 1992) and water *ad libitum*. For the time of the experiment, we observed the general condition of the animals, feed and water consumption on a daily basis and measured their weight on a weekly basis.

We started taking blood from the animals on the eighth week from the experiment start for biochemical, biophysical and immunological tests. Then the animals were devitalized under aether anaesthesia. The weight of visceral fat was measured.

Morphological liver study was performed to define the steatosis stage. For that purpose the liver was fixed in a 10% neutral formalin solution, following which it was passed through graded alcohols with further being submerged into paraffin in accordance with the standard practice. The main histological changes were studied after haematoxylin-eosin staining, for the purpose of collagen detecting, the paraffin samples were stained using a Van-Gieson's method.

The obtained histological preparations were studied using an Olympus BX-41 microscope with further morphometry using the Olympus DP-Soft (version 3:1).

For the purpose of assessing the ultrasound shear wave elastography, Ultima PA was used by Radmir Company, the Subsidiary of the Scientific-Research Institute of Radio Engineering Measurements JSC, Kharkiv, Ukraine, *in vivo,* contact linear sensor at the 7-10MHz frequencies and 10-30mm depth.

Phantom is an acoustically consistent special device to perform the ultrasound liver elastography of rat liver *in vitro*. The phantom used for the ultrasound liver elastography in rats appears to be a bath filled with an immersion fluid, i.e. a physiological saline solution. The bottom of the bath is covered with a non-echo layer. The echo (reverberations) may cause noise in the measures of the ultrasound liver elastography of rat liver. The bath phantom is of 5x5xcm dimensions and 15cm depth. A rat liver sample was submerged at the depth of 5cm from the immersion fluid surface using a special mesh platform that is acoustically clear and does not originate any acoustic noise, but allows for holding the sample under investigation at a certain distance from the ultrasound sensor. Thus preventing any sample deformation by pressing the ultrasound sensor. Ranges of interests were selected visually for the elastographic studying in the left and right liver lobes. Portal tracts, capsules and portal fissures were excluded from the range of interest. The data were recorded in the digital image archive of the ultrasound sensor of the Ultima PA for further processing, using the DICOM format.

Student's t-test was used for the statistical processing.

RESULTS

It has been established that pathological changes in body tissues are always accompanied by various shear stiffness changes of soft tissues. Modulus of shear stiffness has been introduced in the physics to describe the said type of the elastic properties of the matter. The said parameter may vary by 300-400% in the abnormal tissues and by 1,000% from the standard with tumour formations. Thus and so, standard manual palpation is still used for diagnosis of such abnormalities being a simple and rather reliable diagnosis method. Deformations are created in the organ or tissue under investigation at the time of palpation, including shear giving the information about their condition. In fact, no modulus of shear stiffness can be determined based on the resistance value of soft tissues as a response to the external stress, but rather a so-called Young's modulus. This modulus is used to describe elastic properties of the matter at free contraction or expansion. In terms of medical physics, soft tissues are rubber-like matter.

Hence, there is a proposal to use shear waves instead of compressional plane ultrasound waves for diagnosis of abnormal formations. The very shear wave that is determined directly with the modulus in shear will reflect from non-homogeneous inclusions.

Measuring of tissue movement under the load including further restructuring of special distribution of modulus of shear stiffness is common to all modern techniques. These techniques are similar to palpation by tissue compression, where palpation also employs external mechanical load.

We have used for our work a domestic scanner ULTIMA by Radmir (Kharkiv) where a one-dimensional tissue stiffness visualization and assessment of Young's modulus by means of shear wave elastography is used.

Colour pixels are displayed on the ULTIMA RA screen in the range of interest according to the colour scale to measure tissue stiffness in kPa (blue – soft, red – stiff) and digital stiffness values within the control volume (mean, min, and max).

No abnormal changes were revealed by shear wave elastography of the left and right lobes of 8 healthy rats (Fig. 1). Increased liver stiffness values were revealed by the shear wave elastography of the left and right liver lobes of 12 rats belonging to the experimental group (Ta-

ble 1, Fig. 2). Morphologically, fine-drop fatty infiltration of liver pulp hepatocytes without any signs of inflammatory and fibrous changes was found in the experimental group (Fig. 3–6). The fact is an indication of the sensitivity of the shear wave elastography technique to reveal pure steatosis in the rat liver.

Table 1. Parameters of ultrasound shear wave elastography in the control and experimental groups of animals

	Control group (n=8) (kPA)	Experimental group (n=12) (kPA)
Right hepatic lobe	4.52 ± 0.97	6.94 ± 1.9*
Left hepatic lobe	3.89 ± 1.13	6.21 ± 1.02*

p<0.01

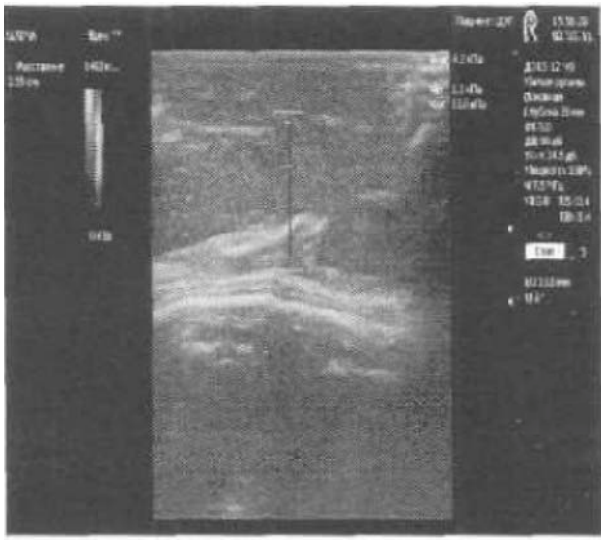

Fig. 1. Shear wave elastography of the liver of a healthy rat

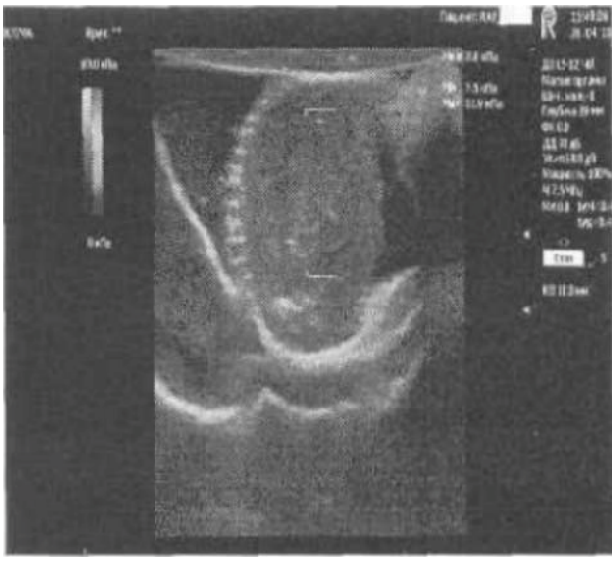

Fig. 2. Shear wave elastography of the liver of a rat suffering from steatosis

The above-mentioned issues are indications of the fact that the present technique can be applied as non-invasive markers for diagnosis of the non-alcoholic steatohepatosis.

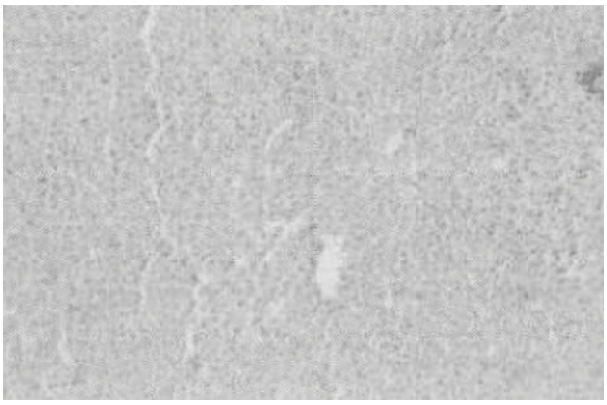

Fig. 3. Van-Gieson's staining, no fibrogenesis intensification observed, few thin threads of connective tissue stretching from portal tracts

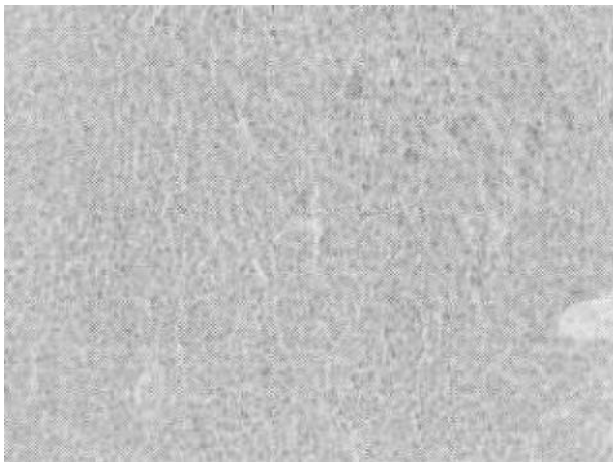

Fig. 4. Balk structure of liver lobe displayed, moderate infiltration with mononuclears observed (signs of acute inflammatory response), vessel congestion, and fine-drop liver steatosis

DISCUSSION

The modern visualization techniques, including ultrasonic diagnosis, CT and MRI, allow for steatosis identification (provided more than 1/3 of the liver has been affected), however they are not reliable for the NAFLD or fibrosis diagnosis [4,5,6,9,11].

Echodensitometry, being an ultrasound diagnosis technique used for diagnosis of the diffusive fatty hepatosis and having some advantages over the puncture biopsy due to it non-invasiveness (which allows for its application as a screening technique to determine the diffusive fatty hepatosis and case follow-up of the morphologic changes of the liver structure over the treatment). The technique implies the ultrasound diagnosis of the patient and diagnosis of the diffusive fatty hepatosis by means of combining such parameters as diffusive increase of the echogenicity of the liver pulp, deterioration of the ultrasound signal in the liver areas located deep and increase of the liver size [10].

The target is obtained by means of performing the echodensitometry of both zones of the patient's liver and

calculating and assessing the objective parameters of the histogram and defining the degree of manifestation of the diffusive fatty hepatosis based on changes of the said parameters vs. the standard values.

The novelty of the invention consists in use of the ultrasound that allows for assessing the parameters of the echodensitometric curves and the severity level of the fatty hepatosis.

The said technique does not lack any disadvantages as long as its sensitivity and specifics is high in case of diffusive organ affection only that is not always the case in the clinical practice. To the contrary, according to the recent morphological research, non-homogeneity of the fatty infiltration of the liver pulp of various manifestation degrees of the histological changes is typical. Secondly, computer-aided and computational processing of the data received from the ultrasound diagnosis parameters is required thus limiting its full application. Thirdly, it renders impossible to clearly define the quantitative content of the fat in the liver and its topographical localization. It will be also observed that the accuracy of selection of the ranges of interest depends on the doctor's qualification and it is of subjective nature thus affecting the interpretation of the obtained data. Absence of calibration of the acoustic settings of a certain ultrasound device with a metrological phantom which affects the echogenicity of tissues on the image is the main disadvantage of the echodensitometry being a digital study technique.

The accumulation of fibrous or fatty tissue in the liver causes the alteration of the physical properties of the liver. They can be supposedly divided into direct and indirect techniques. For example, direct techniques include defining blood velocity in the portal system that depends on the liver density. According to the research performed by Liu and co-authors, a pulsitile index of the splenic artery and average blood velocity in the portal vein are the most informative in terms of defining the manifestation of the liver fibrosis for the Doppler test [5]. The techniques of defining stiffness or elasticity of the liver to the contrary (elastography) are the most perspective at the moment for clinical application. The perspective of development of the said techniques is due to employment of ultrasound equipment available at most centres.

The ultrasound liver elastography using FibroScan (EchoSens, France) is the most popular among the direct techniques of defining the liver stiffness [13]. The liver is studied through the intercostal space. The technique is based on the Hooke's law that states that the extension of a spring is in direct proportion with the load applied to it. The sensor is made of a receiver piezoelectric cell and LF generator mounted on a single axle. A pulse-echo ultrasound technique is used in the process of studying to detect the propagation of the created mechanical oscillations onto the substrate organ tissue and define their velocity. The velocity depends on the tissue density: the denser the tissue the faster the wave propagates. Thus, the density (elasticity) of the liver tissue is measured in kilopascals (kPa). The liver density is in proportion to the square velocity of the wave propagation. According to 7 large European researches involving over 2 000 patients suffering from the chronic viral hepatitis B and C, the sensitivity and the specifics of the ultrasound elastography in detecting the fibrosis of the IV stage (cirrhosis) constituted over 90% [13]. The diagnostic value of the technique has not been fully defined for the NAFLD [6]. The metabolic syndrome can affect the liver density and even in the absence of the biological markers of the NAFLD. According to the recent research involving 429 patients without any liver abnormality and standard levels of the liver enzymes, increase in the liver density was observed on elastograms in the patients suffering from the metabolic syndrome (n=59) as compared to the control (61.5±1.6 vs. 5.3±1.5 kPA respectively đ.0001) [8]. As long as the liver area that is examined using the ultrasound elastography is shaped as ca. 1*4cm cylinder (ca. 1/100 of the whole liver pulp) of 25-65mm depth from the skin surface and is limited with the edge of the right lobe of the right liver lobe, there is a risk of the sample error. The probability of the technical error of the ultrasound elastography is by 9 times bigger against the manifested abdominal fattiness (body weight index over 28 kg/m2) [5].

The limited anatomical area of the liver that is available for the study and the absence of any visual navigation of propagation of the acoustic beam sensing the liver are significant disadvantages of the ultrasound liver elastography using the FibroScan.

With view to the above defects of application of the modern visualization techniques the proposed technique of the ultrasound liver elastography in rats using the Ultima PA sensor (Radmir Company, the Subsidiary of the Scientific-Research Institute of Radio Engineering Measurements JSC, Kharkiv, Ukraine, *in vivo,* contact linear sensor at the 7-10MHz frequencies and 10–30mm depth differs from the similar devices by the following: the ultrasound liver elastography is performed on-line together with the regular ultrasound examination thus allowing for the visual navigation of the range of interest of the elastography of any liver areas. The shear wave elastography being a physical principle does not require any extra physical (acoustic or vibration) load onto the tissues under investigation. The absence of the compression of the liver sample allows for the ultrasound liver elastography of rats in vivo and in vitro using a special acoustically consistent device – the phantom. The ultrasound liver elastography gives the qualitative information (one-dimensional colour mapping using a calibrated scale) and quantitative information (tissue stiffness values measured in kilopascals of the calibrated scale) from the range of interest.

All the above creates the favourable conditions for the application of the shear wave elastography as a non-invasive diagnosis marker for the non-alcoholic steatohepatosis.

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