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MD THESIS

THE HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF HEPATIC STEATOSIS IN VIRAL CRONIC HEPATITES AND NON-ALCOHOLIC STEATOSIS

ABSTRACT

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Introduction

Hepatic steatosis is a partially reversible characterized by the accumulation of triglycerids drops (vacules) in the hepatic cells. Fats accumulation in the hepatocytes cytoplasm is a complex physiological and morphopathological process, where there intervene a multitude of etiopathogenic factors that alter the fats metabolism (Reddy JK, Rao MS, 2006). The condition prevalence reaches 17% in China, up to 30% in the United States (Browning JD, Szczepaniak LS, Dobbins R et al, 2004; Fan JG, Zhu J, Li X J et al, 2005).

There imposes the diagnosis of steatosis when the liver fat exceeds 5-10% of its weight (Crabb DW, Galli A, et al 2004; Adams LA, Lymp JF, et al, 2005).

The most common factors involved in the etiopathology of the hepatic steatosis are alcohol intake, obesity, dislipidemias and diabetes melliuts. From the clinical and histopathological points of view, it is difficult to make a distinction between the alcoholic fat liver and the non-alcoholic fat liver, both conditions being characterized by the accumulation of lipidic vesicles of various sizes in the hepatocyte cytoplasm. Fat accumulation in hepatocytes may also be accompanied by a progressive inflammation of the liver (hepatatis), a clinical condition histopathologically called steatoheptatis.

Regarding the relation between steatosis and cronic hepatitis, numerous authors remarked that hepatic steatosis is frequently met in the patients with cronic C heptatis (from 30 % up to 70% of the patients), when the cronic heptatis infection is diagnosed (Cholet F, Nousbaum JB, Richecqeur M, et al , 2004; Cama C, Bruno S, Di Marco V, et al, 2006; Wu JY, Chen LS, Qiang WG, 2006). Hepatic steatosis was recently identified as a risk and progression factor of extensive for the development of hepatocellular carcinoma (Adinolfi LE, Gamberdella M, et al, 2001; Asselah T, Rubbia-Brandt L, et al, 2006; Lonardo A, Loria P, et al 2006). Other clinical studies indicate that hepatic steatosis may have a negative impact upon the evolution of the disease and response to the treatment of the cronic C hepatitis (Patton HM, Patel K, BehlingC, et al. 2004; Castera L, Chouteau P, Hezode C et al, 2005; Patel K, Zekry A, McHutchison JG 2005).

CHAPTER I. Histophysiology of the Liver

The liver is the largest glandular tissue in the body, representing approximately 2.5% of body weight. Embriologically speaking, the liver develops from the endoderm wall that forms the hepatic diverticulum. The cellular proliferations at this level give birth to hepatocytes that will arrange in cell cordons that will from the hepatic parenchyma. Also from the hepatic

diverticulum there develops the common bile duct out of which there will develop the bladder and biliary ducts.

Until recently, the histological structure of the liver has been dominated by Malpighi's lobular concept, according to which the hepatic lobe is the morphofunctional unit of the liver. The lobe is attributed a hexagonal form, having the center-lobular vein in the center, where the veins from the perilobular network arrive; in the loops of this network there are found the hepatocellular cordons, made up of two rows of radially placed cells. At the junction point of various lobes, there form the portobilliary spaces or the Kiernan spaces, where there are placed the blood and lymphatic vessels, the billiary ducts and the nerves.

Nevertheless, recent research studies, based on the most modern technology, show that the morphofunctional unit of the liver is the hepatic acinus, made up of ab inform mass of hepatocytes, placed around a portal venule (the axial vein). A group of 2 -3 acini tributary to an axial vein makes up a complex ancinus. The hepatocytes located close to the terminal branch of the axial portal venule makes up the first area of hepatocytes (metabolically active); the hepatocytes situated at the ancinus level form the third area (cells adapted to the storage function); between them there is found the second area, with hepatocytes that make glycogen exchanges between the areas.

According to Eppinger's diagram, there were hepatocellular cordons placed in two rows, between them being the biliary canaliculi, which heads towards the sinus at the periphery; between the hepaticytes and the sinus there are situated the Diesse gaps; in this way, the hepatocyte was attributed two poles: a vasculary one and a billiary one. According to the ancinous concept, there are unicellular lamellae that transpass each other, with blood vessels in their crossings. In this way, the hepatocyte is bathed on 2-3 sides by sinusoids, while on the other sides it comes into contact with 2-3 biliary canaliculi.

Hepatocytes represent 80% of the total cells at liver level. They have a polyedric shape and a 20-30 μm diameter. Their lifetime is approximately of 150 days. In optical microscopy, hepatocytes may be uni- or binucleated, most of them being uninucleated.

According to their localization within the three areas, hepatocytes present a metabolic gradient, size, enzymatic activity and organites content, also depending on the cell localization within the acini (MacSween RNM, et al, 2007).

In area 1 there are the hepatocytes receiving the blood with the highest oxygen quantity, being close to the perilobular artery. These hepatocytes have more characteristics: they are the most metabolically active, they are the first to deposit glycogen postprandially; they are the first to signal the regeneration signs in case of hepatic lesions; they are the first to be subject to the

action of toxins absorbed in the digestive tube, and in case of biliary stasis, they are the first to suffer any alterations.

In area 3 hepatocytes are also subject to toxin action, but in a smaller quantity. Here arrives the blood with the lowest oxygen content. That is why, in ischemias, the first to suffer are the cells in area 3 (the cells in the vicinity of the center-lobular vein).

In area 2 the cells have intermediary characteristics to the other two areas.

Ito cells (lipocytes, striated cells, interstitial cells) are found quite rarely (an Ito cell at 20-25 hepatocytes). They have numerous lipidic drops. They are in strong relation with the conjunctive fibers in the Disse space. Also, part of them surround the sinusoid capillaries. They come into contact with the hepatocytes, without realizing any junctions. They may transpass the hepatocyte cordons. They play various parts: they secret collagen type 2 that organizes itself into reticulin fibers, the growth factors of hepatocytes, they store exogen lipids and Vitamin A; during fetal life, they intervene within hematopoiesis.

'Pit' cells may be identified only under the electronic microscope. They are similar to the endocrine cells; in the cytoplasm they have electrodense granules with a 300 nm diameter. That is why, at first, they were considered to play an endocrine role. Nowadays they are considered a variety of circulating lymphocyes, being large lymphocytes, with granulations and killer activity.

Kupffer cells – they derive from the circulating monocytes and belong to the mononuclear phagocytic system. They are located at the junction between two sinusoid capillaries and bulge towards the lumenus. They have a stellated shape, an euchromatic nucleus and present various organelles than the endothelial cells. They have various characteristics: they have organelles involved in the syntheses (RER, mitochondria), because these cells synthesize part of the liver export proteins, also contain organelles involved in the processes of cytoplasmic degradation (lysosomes, phagolysosomes and residual bodies), are involved in the immunity through their capacity of phagocytosis, they have receptors for immunoglobulins and complement, they secrete cytokines, etc.

CHAPTER II. Viral Chronic Hepatites – Clinical and Histopathological Evaluation

Cronic hepatitis is term that is histopathologically defined as the persistance of the inflammatory reaction of the liver and clinically defined as the persistance of clinical signs and alteration of biological samples for more than 6 months.

The histopathological alterations in cronic hepatites are highlighted through hepatic puncture. In cronic hepatitis, the portal gaps may be normal or of high dimensions due to the

presence of the inflammatory infiltrate made up of lymphocytes, rare plasmocytes and sometimes segmented leukocytes. In hepatitis C, the lymphoid aggregates may form lymphoid folicles with germinative centers. The inflammatory infiltrate may be mild, moderate or severe. This infiltrate includes mainly positive CD4 lymphocytes T helper and plasmocytes; more rarely, there are also descibed macrophages. The inflammatory infiltrate may extend into the hepatic lobe, thus producing the erosion of the interface area. The hepatocyte necrosis may be focal, involving individual cells or groups of cells. The most severe form presents extended areas of necrosis confluent with the isolation of certain hepatocyte groups, in a rossette shape. Confluent necrosis that joins the vascular structures is called bridged necrosis; it may appear between the portal gaps and the terminal veins.

CHAPTER III. Interpretation of Hepatic Biopsy

Liver biopsy has become a safe and rapid method, a standard procedure of investigation and monitoring of the patient with cronic hepatitis.

In vacuolar degeneration (hydropic inflammation of the liver), the hepatic cells are oversized, the cytoplasm is rare, dark-pale, vaculoized or empty, with cytoplasm material droppings, granular or stratified. The most affected are the hepatocytes at the periphery of the hepatic ancinus (3rd area) (Mihm S. 1997).

The lithic necrosis of hepatic cells is associated with the hydropic inflammation of the cytoplasm and nucleus, the decomposition of cell exterior and of nuclear membranes. The necrotic cells suddenly drop-out. In contrast with acidophilic necrosis, lithic necrosis has a short life. The droppings are rapidly phagocyted by the macrophages and by the Kupffer cells. Lithic necrosis is mainly located in the perivenular regions.

The acidophilic degeneration is the coagulation type degeneration of the hepatic cells, with pronounced eosinophilia, reducing of the cytoplasm and nucleus pyknosis. Most of the time, cells have a rhomboid contour. The nucleus disappearance, and its elimination from the “platform like” cells, leads to the formation of acidophilic Councilman bodies, difficult to phagocyte.

Focal necrosis is the individual necrosis of the hepatic cells or of a small group of adjacent hepatic cells. Focal necrosis may be sometimes located in the lobule. Most often, focal necrosis has a short life, of the “short-live” (lithic) type, and is surrounded by a lypmh histocytarian infiltrate placed in small agglomerations.

The “piece-meale” necrosis was first described as an immunological type of the hepatic cell necrosis by Popper & Co. (1965). It may be defined as a cronic inflammatory, gradual

destruction, of the isolated cells or of small groups of hepatic cells at the mesencgyme-parencyma interface (periportal, periseptal or alongside the confluent necrosis), associated with an inflammatory lymph histiocytarian infiltrate.

The “Lafora” bodies are intracytoplasmic inclusions, with a predilection for periportal hepatocytes very similar to that of “groundglass” hepatocytes. They are composed from an unusual branched polysaccharide and they are positive in the PAS staining, as well as in the carmine, Lugol and colloidal ferrum staining, and it does not stain with orceine or adheide-thionin.

The bilirubin stasis, easily to recognize in the conventional haematohylin-eosine or ferrum staining, shaped as: canalicular bile trhombus, in a perivenular dsiplacement in early stages, with a change especially towards the periferrolobular regions in advanced stages; the intracytoplasmic granules of the biliary pigmentation, in hepatocytes and/ or the Kupffer cells; storage of ductular and ductal bile, the latter being very rarely observed in biopsy samples.

CHAPTER IV. Purpose and Objectives of Thesis

The purpose of our study was to assess various clinical, paraclinical, histological and immunohistochemical aspects in the patients with hepatic conditions, in whom there was performed a hepatic biopsy puncture for diagnosis and for the correlation of histopathological aspects of steatosis with the evolution of hepatocytarian lesions and with the clinical evolution of patients.

We proposed the follwing **objectives**:

- identification of steatosis lesions and their classification according to severity;
- correlation of hepatic steatosis with the age and sex of patients;
- correlation of steatosis stage with the necrotic-inflammatory activity;
- correlation of steatosis with the collagen fibrillogenesis process;
- immunohistochemical study of the inflammatory infiltrate associated to steatosis;
- study of tumoral necrosis factors associated to seatosis;
- immunohistochemical study of the fibrillogenesis process;
- study of the reaction of Kupffer cells and of Ito cells in the patients with steatosis.

CHAPTER V. Material and Methods

The material studied in this paper was represented by a number of 628 liver fragments sampled through puncture-biopsy from the patients with cronic hepatitis or suspect of any cronic hepatic condition. The biological material was fixed in 10% neuter formalin solution and

processed by the paraffinum inclusion technique, after which we performed usual stainings with hematoxylin-eosine (HE), van Gieson, as well as histochemical stainings of the Gomori argentic impregnation for reticulin or the PAS-hematoxylin staining. For the immunohistochemical study there were used the following antibodies: CD20, CD45ro, CD4, CD68, alfa-actina, TGF-beta.

CHAPTER VI. Histopathological and Immunohistochemical Study of Hepatic Stenosis

The study was performed over a period of 6 years (2006-2011) on a group of 628 hepatic biopsy puncture, out of which there were selected for study 306 cases that presented various steatosis stages. If in the age decade of 20-29 years old there were 20 patients, in the age decade of 30-39 years old there were 45, in the age decade of 40-49 years old there were 85, while in the age decade of 50-59 years old, 132 patients. The age most affected by hepatic steatosis associated to cronic hepatites was between 50 and 59 years old (the 4th life decade) that represented about 43% of the cases. Most of the patients were diagnosed by chance, at a routine abdominal echographic examination, or as a result of various blood tests, or during a medical consult for other causes than hepatic ones. The symptoms in advanced hepatic steatosis were represented by physical asthenia, tiredness, postprandial sleepiness, epigastric pains or discomfort (or in the right hipochondrium), abdominal gases, etc.

Steatosis in pacients with cronic C heptatitis. In the subgroup of patients with hepatitis C virus, the age varied between 20 and 68 years old (the mean age being of 51.8 years old). The age group with most cases was that of 50-59 years old. The most cases of steatosis associated with virus C infection were observed in the age group of 50-59 years old (124 cases, representing 47.87% of the total of cases); the most cases were observed in the female patients.

In the present thesis we studied, in the hepatitis C virus context, the relation between the necrotic inflammatory activity and steatosis and we established that, most frequently, this activity was mild and moderate that correlates with a reduced stage of steatosis. A mild activity characterized by the presence of a lymph plasmocytarian infiltrate in the portal and periportal gaps, in one or more portobiliary gaps, was diagnosed in 97 cases, representing 37.45% of the total of cases. Most frequently, it has been accompanied by mild steatosis (56 cases), 16 cases of moderate steatosis and 25 cases of severe steatosis.

Most cases of steatosis were associated with a moderate inflammatory activity (101 cases, representing 39%). Out of the cases of moderate inflammatory steatosis, the mild steatosis forms were represented by 58 cases (57.42%), moderate steatosis by 20 cases (19.80%), and severe forms by 23 cases (22.78%).

Hepatic fibrosis associated with steatosis, identified in our cases, was quantified from F1 to F4 (cirrhosis) and was correlated with the presence of various stages of steatosis. Mild steatosis (F1) is characterized by the presence of a fibrous expansion of the portal-biliary gaps. In our study, in 60 cases portal fibrosis was without any formation of septa. In most cases, fibrosis was correlated with mild steatosis (33 cases), with moderate steatosis in 19 cases and severe steatosis in 8 cases. Septal fibrosis (F2) was present in most of the cases (91) and was correlated with mild steatosis in 53 cases, with moderate steatosis in 23 and with severe one in 15 cases. Stage 3 of fibrosis, constituted of thick fibrous septa located in portocentral bridges, was found in 80 cases and was accompanied by mild steatosis in 32 cases, by moderate steatosis in 23 and by severe one in 27 cases. Cirrhotic nodules and pseudonodules (F4) were found in 28 cases and in most cases (11) they presented severe steatosis.

Another correlation we made was between the necrotic inflammatory activity in hepatitis and necrosis. The distribution of inflammatory cells varied from one case to another, but all the cases were characterized by the presence of a dense monocytarian infiltrate at the level of portal gaps. The inflammatory infiltrate was made up of lymphocytes and plasmocytes. In cronic C virus hepatitis with mild necroinflammatory activity, mild steatosis predominated (46 cases), being followed by severe steatosis (25 cases) and moderate one (18 cases).

Hepatic steatosis associated with cronic B hepatitis. In the subgroup of patients with B virus hepatitis, there were recorded 47 cases; their age varied between 21 and 64 years old. The F/M sex ratio was of ½. The age group with most cases was situated in the interval of 41-50 years old (28 cases). Steatosis was assessed as mild (0-30% of hepatocytes) (21 cases), moderate (30–60% of hepatocytes) (16 cases) and severe (>60% of hepatocytes) (10 cases).

The study of the necroinflammatory activity showed that the majority of portal gaps presented a variable lymph plasmocytarian infiltrate. The quantification of the inflammatory infiltrate highlighted the following results: stage 1, 17 cases (mild inflammation), stages 2, 3, 11 cases (moderate inflammation), stage 3, 14 cases (marked-moderate inflammation), stage 4, 5 cases (marked inflammation).

Out of the range of found alterations, a relatively specific aspect was highlighted in 41 cases. In these cases, the hepatocytes presented cytoplasmic partial or total alterations, this becoming finely granular, pale, frequently separated by the membrane through a halo. This aspect is known as ”mat galss aspect”. At the level of these cells nuclei there was identified the presence of some central part, finely granular, eosinophils (“sandy nuclei”).

Non-alcoholic steatosis (NASH) is the progressive form of the hepatic lesion that involves a risk for progressive fibrosis, cirrhosis and liver failure.

Non-alcoholic steatohepatitis (NASH) is part of a serious diseases called non-alcoholic fat liver diseases (NAFLD) characterized by an excesive accumulation of fats in the liver, hepatocytarian lesions that go from apoptosis to necrosis, inflammation and hepatic fibrosis, which clinically leads to various stages of liver failure.

It is estimated that approx. 30% of the adult population in developed countries present NAFLD. NASH appears only in approx. 3% of the general population and in 2/3 of the persons with morbid obesity and diabetes mellitus type 2. Its prevalence may, though, increase up to 57% in obese persons, 70% of those with diabetes mellitus, 90% of the subjects with morbid obesity.

The study included 39 histopathological lesions from patients with insulin resistant diabetes mellitus type 2 (37 cases) associated with obesity and high blood pressure and 2 cases from female patients whom were administered birth control pills and antiinflammatory drugs for a longer period of time. The stage of macro and microvesicular steatosis was classified as follows: stage 1 steatosis, where the affected hepatocytes were between 0-33%, stage 2 between 33 and 66%, stage 3 steatosis where there were affected more than 66% of hepatocytes. In our study, 14 patients were diagnosed with mild steatosis, 18 with moderate steatosis and 7 patients with severe steatosis. Hepatocytes balooning, a characteristic indicating cellular damaging, was also identified in 69.8% of cases.

Regarding fibrosis, in our study there was noticed that, at first, this appeared in the center-lobular region and was characterized by its pericellular and perivascular location.

Immunohistochemical study. For the study of the inflammatory infiltrate, we used the CD20 markers for the B lymphocytes and CD45-ro and CD3 for the T lymphocytes. The macrophages and Kupffer cells were highlighted with the specific CD68 antibody, while the myofibroblasts and the Ito cells with the alfa-actin of the smooth muscle.

The inflammatory infiltrate was mainly made up of lymphocytes and plasmocytes; the T lymphocytes were more numerous in comparison to the B lymphocytes, both in the portal and the intralobular gaps, suggesting the important role that T lymphocytes play both in the necroinflammatory process and in the apoptosis induction. Most of the inflammatory infiltrate cells were identified in the conjunctive tissue from the Kiernan portal-biliary gap, diffusely disseminated or forming lymphoid aggregates and lymphoid follicles, with reactive centers.

The distribution of the alfa-actin of the smooth muscle (alfa-SMA) in the patients with mild fibrosis was mainly perivenular. In the group of patients with moderate fibrosis, the alfa-actin expression was intense in the portal gaps and in the septa and weaker perivenularly and in the intermediate gaps. In the cases of severe fibrosis and hepatic cirrhosis, alfa-actin was intensely positive in the portal gaps and in the fibrous septa. The Kupffer cells appeared enlarged

and placed in aggregates in the sinusoids adjacent to hepatocytes with a severe balooning and Mallory bodies.

CHAPTER VII. Discussions

Hepatic steatosis may also be caused by the interaction of various factors, such as viral infections (hepatitis C) associated with the metabolic syndrome and with a large intake of alcohol (20g/day of alcohol) (Crabb DW, et al, 2004). Hepatic steatosis, both alcoholic and non-alcoholic, starts as a simple steatosis; if the cause persists, this steatosis invariably progresses to steatohepatitis, hepatic cirrhosis and even hepatocarcinoma (Zafrani ES, 2004).

In our study, viral hepatic steatosis represented 46.81 % of the studied hepatic biopsies, most frequently being found in the patients with viral cronic C hepatitis (51.34% of viral steatoses). In literature, the general prevalence of steatosis in the patients with cronic infection woth hepatitis C virus (HCV) is of 55.5% (between 34.8-81.2%) (Adinolfi LE, et al, 2001). The mean age of patients was of 51.8 years old, with a F/M ratio of 1,2/1.

As demonstrated by some studies (Hwang SJ, Luo JC, Chu CW, et al, 2001), the assessment of the histological activity index (HAI) is very important as this is correlated to steatosis. In our study, the HAI index below 7 was found in 31.3% of the cases and was correlated with stage 1 (mild steatosis), in 44.1% of the cases; a HAI index over 7 was correlated with moderate and severe stenosis, which represented 55.9% of the cases.

Regarding the impact of hepatic steatosis upon the necroinflammatory activity and upon the hepatic fibrosis process, it is difficult to assess in the patients with cronic C hepatitis, as these processes are multifactorial. Still, various clinical studies and experimental research studies have shown that hepatic steatosis accelerates the development and progression of fibrosis in cronic C hepatitis (Castera L, Hezode C, Roudot-Thoraval F et al, 2003; Browning JD, Horton JD, 2004). We showed that most of the patients presented mild to moderate forms of steatosis, which were associated with a necroinflammatory activity and with moderate fibrosis. A relatively small percentage of patients (10.42%) presented severe steatosis and an intense activity; the percentage of severe steatosis and marked fibrosis was reduced, as well (4.25%).

CHAPTER VIII. Conclusions

The fats accumulation in the hepatocytes cytoplasm is a complex pathological process, where there intervene a series of etiopathogenic factors that alter the fats metabolism. In this condition onset, diet represents an essential element, steatosis being the result of an imbalance between triglycerides accumulation and metabolization. For a long time, steatosis was

considered as a lipid accumulation with no pathological importance. The last decades study have shown that hepatic steatosis is a disease that can lead to steatohepatitis, progressive liver failure and even to hepatic cirrhosis.