ELECTRON MICROSCOPY OBSERVATIONS OF THE HEPATIC TISSUE ULTRASTRUCTURE AFTER THE ACUTE INTOXICATION WITH ETHANOL AT RATS

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Summary
Ethanol is a widely applied solvent used in industry, for consumption, and as a disinfectant agent. Chronic administration of ethanol to experimental animals leads to neurological disorders and organ dysfunctions concerning the alimentary tract, liver, kidneys, heart, and pancreas, accelerates atheromatous changes, and causes alcoholic damage of the foetus. The scientifically research were realized on the Laboratory of Electron Microscopy of Ovidius University and the laboratories and the biobases of Cell and Molecular Biology and Animal Physiology Disciplines on the Faculty of Natural and Agriculture Sciences during April 2009. The objective of scientifically research was to establish the hepatic tissue ultrastructure after acute intoxication with ethanol 50% on Wistar rats. The scientifically research were realized on the female Wistar rats (n=8) and the animals was separated into lot of martor animals and another with experimentally animals. At experimentally rats was administrated a 2 ml of ethanol 50% for 5 days for each animals (5g/kg bw). The slaughtered of animals was been after 6 days after ethanol administrate and was recovery hepatic tissue for histological and electron microscopy examinations. In hepatocytes from experimentally rats were observed a moderate accumulation of lipids. The hepatic tissue had an adipocitar aspect and the hyperchromic nucleus with peripheric chromatine had a central position.

General analysis realized on the hepatic tissue recovery from experimentally animals shown more important structural aspects: hyperplasia of rough endoplasmic reticulum and hyperplasia of smooth endoplasmic reticulum, degeneration of Golgi apparatus, degeneration of mitochondria and numerous lipids accumulations into cytoplasmic matrix. The lipids accumulation was presented into membrane vesicles with different size, between 100 - 900 nm or bigger and disseminated in cell matrices. The number of mitochondria was small and had an moderate optical density and the crests are very dificult to observed. Were observed an irregular inclusions but strong osmophile not included into membranes. The origin is probable to be deed mitochondria, not degreed and not eliminate by intern lysosomes system.

Key words: hepatocytes, ultrastructure, rats, electron microscopy, ethanol

Introduction
Ethanol is a widely applied solvent used in industry, for consumption, and as a disinfectant agent. From 2% to 10% of the consumed ethanol is excreted in an unchanged form by the kidneys and lungs. The remaining amount undergoes oxidation, mainly in the liver. Ethanol particles are small and hydrophilic; this is why ethanol is quickly absorbed from the alimentary tract and from the alveoli. A small amount of it is already absorbed in the oral cavity. The ethanol easily passes through the skin and placental barrier. The biotransformation of 85%-90% of the ethanol proceeds in the organism with the participation of the liver enzymatic systems: alcohol dehydrogenase (ADH), microsomal ethanol-oxidisation system (MEOS), and catalase.

Chronic administration of ethanol to experimental animals leads to neurological disorders and organ dysfunctions concerning the alimentary tract, liver, kidneys, heart, and pancreas, accelerates atheromatous changes, and causes alcoholic
damage of the foetus (Matsumoto and Matsumoto, 2008). Ethanol intoxication is considered to be the main risk factor for the occurrence of a chronic pancreatitis in 80% of cases (Gullo, 2005; Kono et al., 2001). Ethanol as a possible acute hepatotoxic agent has been the subject of a number of recent investigations (Mallov and Bloch, 1956; Di Luzio, 1958, Lieber et al., 1959; Di Luzio and Zilversmit, 1960). These studies have indicated that, aside from the conventional concept of nutritional deficiency resulting from caloric replacement in chronic alcoholism, an additional influence arises from the acute toxic effects of ethanol itself. Older experiments showed that alcohol administration, even when accompanied by an adequate supply of choline and other dietary requirements, might cause fatty liver in rats. The employment of better controlled approaches, especially the use of isocaloric glucose administration in control animals, has thoroughly established that acute ethanol intoxication produces fatty liver (Mallov and Bloch, 1956; Di Luzio, 1958, Lieber et al., 1959; Di Luzio and Zilversmit, 1960; Ikejima, 1998; Lieber, 1980; Monden, 1991; Naveau, 1997; Pinzani, 1995). It has recently been observed in man, as in experimental animals, that isocaloric replacement of carbohydrate by alcohol will produce a fatty liver despite adequate dietary intake, supporting the concept of the toxic effect of ethanol (Lieber et al., 1963). The metabolic effects of alcohol on the liver have recently been reviewed (Lieber, 1966; Isselbacher and Greenberger, 1964). Most of the recent inquiries into the pathogenesis of the alcohol-induced fatty liver have been concerned with the biochemical derangement which occurs. What this defect or defects may be has not yet been established. The authors (Horning et al, 1960; Brodie et al., 1961; Zang, 1997) suggested that acute alcoholic intoxication in rats caused increased mobilization of fatty acid from adipose tissue stores, which resulted in excessive accumulation of fat in the liver. Lieber and Schmid (1961), presented evidence indicating that increased hepatic fat content was due to increased concentrations of NADH₂ within the hepatic cell, which caused increased triglyceride synthesis. Nikkila and Ojala (1961) demonstrated that incorporation of palmitic acid into liver triglyceride increased 4 hour after ethanol administration, and postulated that even when the plasma free fatty acid level is not elevated an increased formation of hepatic triglyceride is an important factor in the pathogenesis of the ethanol-induced fatty liver. Reboucas and Isselbacher (1961) concluded that the acute ethanol fatty liver was the result of impaired lipid transport.

Elko, Woole, and Di Luzio (1961) and Poggi and Di Luzio (1964) studied lipid mobilization from epididymal fat pads during acute ethanol intoxication. They found no increased fatty acid release from adipose tissue during ethanol intoxication and their studies indicated that the hepatic steatosis was possibly due to depression of triglyceride metabolism by the liver cells. Recently, Lieber et al (1963) have also reported that fatty liver developed in man and rats before there was any indication of decreased hepatic triglyceride release or excessive peripheral fat mobilization. During the accumulation of liver triglyceride following ethanol administration there is essentially no alteration in the plasma triglyceride level. However, when triglyceride is administered to ethanol-treated rats, hypertriglyceridemia develops (Enomoto, 1998; Umuro, 1997; Johnson et al., 1992; Thurman, 1998). The scientifically research were realized on the Laboratory of Electron Microscopy of Ovidius University and the laboratories and the biobases of Cell and Molecular Biology and Animal Physiology Disciplines on the Faculty of Natural and Agriculture Sciences during April 2009. The objective of scientifically research was to establish the hepatic tissue ultrastructure after acute intoxication with ethanol 50% on Wistar rats.
Materials and methods

1. Animals
The scientifically research were realized on the female Wistar rats (n=8) and the animals was separated into lot of martor animals and another with experimentally animals. The rats had the weights between 200-220 g. At experimentally rats was administrated a 2 ml of ethanol 50% for 5 days for each animals (5g/kg bw). The slaughtered of animals was been after 6 days after ethanol administrate and was recovery hepatic tissue for histological and electron microscopy examinations.

2. Elaboration of histological sections
Hepatic tissue was recovery immediately after rats slaughtered and was introduced in CARNOY fixator for preservation of intracellular structures. After than the pieces of tissue were transferred in 3 butanol baths for 3 hours and were mounted into paraplast. The sections of tissue was realized with the microtome and colored with hematoxiline - eosin or hematoxiline eosin- metilen blue.

3. Elaboration of electron microscopy sections
Small pieces of hepatic tissue (1-1,5 mm) were fixed in MILLONING tampon with 2,7% glutaraldehide, pH 7,2, for 4- 6 hours. The fixation was realized with 1% OS0₄ (osmic acid) during one hour at +4°C. After washing the pieces for eliminates the excess of fixator, these were dehydrated into alcohol bath, propylene oxide and were included into Epon 812. The sections (500-600Å) was obtained with an ultramicrotom ULTRACUT R and colored with uranyl acetate and lead acetate. The sections of tissue were examined and photographed at electronically microscope Philips- 302.

Results and discussion

The study of morphostructural modifications of liver by histological and electron microscopy examination

1. Histological examination
The structure of hepatocytes on martor animals had a normal aspect, in cytoplasm were observed aggregates intense basophile represented by rough reticulum endoplasmic which synthesize the proteins. In hepatocytes from experimentally rats were observed a moderate accumulation of lipids. The hepatic tissue had an adipocitar aspect and the hyperchromic nucleus with peripheric chromatine had a central position.

There were no detectable changes in hepatic cell size, granularity of cytoplasm, or cytoplasm basophile in the experimental group compared with animals receiving glucose and corn oil.

The glycogen reduction was more marked in animals receiving ethanol. Sudan black B stains indicated a definite increase in number of hepatic cell lipid droplets in rats receiving ethanol. The fat droplets were not found in the hepatic tissue of control animal. In the ethanol-treated group fine sudanophilic droplets measuring about 1μ in size were noted. The fat droplets were located mainly in portions of the hepatic cell cytoplasm adjacent to the sinusoidal walls (figures 1 and 2). This alteration was panlobular in distribution, and was observed at the periphery of the lobules. Histochemical stains for succinic dehydrogenase, NAD- and NADP-diaforeses, and ATP-ase showed no reduction in these enzymes in the alcohol-treated animals (Ashworth et al., 1965). Histological studies showed no evidence of marked hepatocellular injury resulting from the effect of acute ethanol intoxication, and there was no gross loss of any of the cytoplasm enzymes. These observations suggested that the resulting cellular disorder and lipid accumulation were related more to the biochemical processes of lipid transport and mobilization than to a structural cellular defect. The effects of ethanol intoxication were observed into hepatocytes because had a numerous lipid droplets (McCalla et l., 1957; Kono et al, 2001).
2. Electron microscopy examination

General analysis realized on the hepatic tissue recovery from experimentally animals shown more important structural aspects: hyperplasia of rough endoplasmic reticulum and hyperplasia of smooth endoplasmic reticulum, degeneration of Golgi apparatus, degeneration of mitochondria and numerous lipids accumulations into cytoplasmic matrice. The rough endoplasmic reticulum had infrequent the hypotrophy aspect and into connexion with hypertrophied smooth endoplasmic reticulum. The glycogen rosettes were in small number or absents. In microphotographs are presents the area with small glycoprotein inclusions.

The laterally inter hepatocytes space were small, and the area of bile was 200-250 nm. The nucleuses were apoptotic, situated easy eccentric at basal pole cell. Cytoplasm may content a small number of collagen and reticulate fibers represented a large champs and necked around the endoplasmic reticulum. The rough endoplasmic reticulum with ribosome does discontinue situated at membranes and cisterns grown. Cisterns of endoplasmic reticulum are easy curved with spaces which delimited the perfect oval area and who continued with smooth endoplasmic reticulum.

Were observed an irregular inclusions but strong osmophile not included into membranes. The origin is probable to be dead mitochondria, not degreed and not eliminate by intern lysosomes system. The lipids accumulation was presented into membrane vesicles with different size, between 100 - 900 nm or bigger and disseminated in cell matrices. In this phase, the glycogen rosettes are absents, exist a small number of mitochondria mostly degenerated and cell matrice (filaments and microtubules). The excessive accumulation of lipids can be form into sacks of endoplasmic reticulums. The number of the Golgi apparatus was small and around are small vacuoles plump with homogen and translucent material.
The hepatocytes are invaded by the lipids not included in vesicles but surrounded by the fibrous. Another form of cell death was presented like nucleus cariolyosis and dispersion of the nuclear bodies in cytoplasm. The hepatocytes was presented an diffuse aspect with a few diffuse and intens osmophile organelles (Zamfirescu, 1999).

At animals fed with ethanol the mitochondria were similar in size and structure with mitochondria from control animals. No abnormalities in the endoplasmic reticulum, ribosomes, or Golgi apparatus were seen. Glycogen was markedly decreased in the cytoplasm in ethanol-treated rats. Numerous complex arrays of fine tubules and non-granular membranes were noted in the cytoplasm. At animals intoxicated with ethanol, the lipid particles and larger lipid droplets were more abundant in the hepatic cell cytoplasm, and the spaces of Disse contained a greater number of small chylomicrons.

There was marked increase in the number of lipid particles in membrane-lined vesicles within the liver cells. In contrast to the control animals, these intravesicular lipid particles were present throughout the cytoplasm, showing little or no tendency to diminish in size and number deeper in the cytoplasm. The larger (1-2 μ) lipid droplets were still larger and more numerous than in controls. Small cytoplasmic vesicles containing lipid particles 500-800 A in diameter were frequently seen in close proximity to or in actual contact with these larger droplets.

Lipid particles in the smaller vesicles sometimes appeared to be in contact with the outer part of the larger lipid droplets. The margins of the larger droplets were frequently irregular and tented in appearance. The hepatic cells of control rats contained numerous cytoplasmic osmophile particles. These were present in approximately equal concentration in all portions of the cell and were quite uniform in size. They averaged 530 A in diameters, and were located in vesicles with not granular membranes. Similar particles were present in these martor animals in the space of Disse and in hepatic sinusoidal blood. In all groups of animals studied, the Golgi vesicles of liver cells were found to contain oval or ellipsoid osmophile particles 400-600 A in size.

These resembled the small osmophile particles inside cytoplasmic vesicles in other portions of the liver cells, except for their ellipsoid shape. Not elucidated the nature of the chemical defect in lipid metabolism which occurs during acute ethanol effect (Ashworth et al, 1965; Spitzer, 1996). At both lots of animals, control and alcohol-treated rats, numerous
small lipid droplets were observed in the sinusoids and in the space of Disse. These droplets are believed to be formed in the intestines and brought to the liver (Ashworth et al., 1960). The time of their appearance in the hepatic sinusoids during the process of intestinal absorption is also compatible with this interpretation of their origin (Zamfirescu, 1999). The increased hepatic triglyceride content in acute ethanol intoxication is accompanied by a marked accumulation of lipid particles in membrane-limited vesicles throughout the liver cell cytoplasm. In other forms of hepatic steatosis a similar deposition of accumulated cytoplasmic lipid occurs (Ashworth et al., 1963; Ashworth et al., 1965).

It could be argued that intravesicular particles represent lipid which has been taken into liver cells from the blood by pinocytosis or that they are lipid containing particles, such as β-lipoproteins, which are being synthesized within the liver cell preparatory to secretion into the blood. It is also possible that hepatic cell intake and output of particulate lipid both occur simultaneously, and that both processes may be represented by intravesicular cytoplasmic lipid droplets. In control rats we observed lipid particles in vesicles located mainly in the peripheral portion of the liver cell cytoplasm, while in ethanol treated animals intravesicular lipid particles were more numerous and more widely distributed throughout the liver cells.

In alcohol-treated animals smaller particles frequently were partially fused to form larger droplets and were often contiguous with very large, storage-type lipid bodies. One explanation for these observations, although it cannot be substantiated by morphological evidence alone, is that in the normal animal lipid usually enters the liver cell within pinocytotic vesicles and is then metabolized, while during ethanol intoxication the intracytoplasmic metabolism and disappearance of lipid particles taken up by liver cells are impaired (Ashworth et al., 1965). The chylomicrons appeared in liver cells without molecular rearrangement (Borgstrom and Jordan, 1959; Reiser et al., 1961; Stein and Shapiro, 1961).

Disappearance of intravesicular lipid particles in liver cells is consistent with the concept of subsequent degradation of triglyceride (Borgstrom and Jordan, 1959; Roheim and Eder, 1961) followed by diversion of resulting fatty acid into biological oxidation (McCalla et al., 1957) its reassembly as stored triglyceride, or its incorporation into lipoprotein for secretion from the liver cell into the blood (Roheim and Eder, 1961). Knowledge of the morphologic appearance and site of formation of β-lipoprotein within the liver cell is a hindrance to the interpretation of the observation of small cytoplasmic lipid particles. Low density β-lipoprotein particles have been reported to have an average diameter of 350 Å (Reiser et al., 1961). In animals absorbing corn oil, the lipid particles which we observed in hepatic cell vesicles measured 500-2500 Å. Therefore, at least the larger particles observed were probably not β-lipoprotein. On the other hand, in both control and alcohol-treated animals which were absorbing triglyceride from the intestine, the increased hepatic cell uptake of chylomicrons lipid would lead one to expect an increased rate of hepatic cell lipoprotein synthesis and secretion. During the process, lipoprotein particles at controls and ethanol-treated animals could very well represent low density β-lipoprotein. The effect of ethanol on the liver increased the intestinal absorption. The fatty acid mobilized from adipose tissue can be the source of increased triglyceride in ethanol-induced fatty liver (Brodie et al., 1961; Reboucas and Isselbacher, 1961; Elko et al., 1961).

Ultrastructure evaluation after intoxication with ethanol of experimentally rats was realised at pancreas level (Pedrycz et al., 2008). In pancreatic preparations coming from rats of the control group,
vesicular cells with large nucleus were observed in the exosecretory part. In the basal part of the cells, amount of the membranes of rough endoplasmic reticulum was observed and in the top part, amount of zymogen grains. In pancreatic preparations coming from rats of the experimental group, the broadening of the rough endoplasmic reticulum cisterns in vesicular cells was observed. In the neighbourhood of basal membranes of these cells, the accumulation of the bands of collagen fibers was observed. In the area of the nucleus, were present large clusters of heterochromatin that accumulated in the central part of the nucleolus (Kot et al, 2008). In literature, it is shown that already the first dose of ethanol causes an increase in cellular membranes liquidity, which favours the later accumulation of cholesterol in the basal membrane. Successive doses of ethanol fix cellular membranes leading to the occurring of biochemical changes and the decrease of activity of Na⁺/K⁺ATP-ase (Rothman et al, 1996).

Conclusions
The scientifically research realized regarding the effects of acute intoxication with ethanol at the female Wistar rats were permitted to formulation a few conclusions like:
1. The dose of ethanol administered on the experimentally rats was 5 g / kg bw and was induced the morphostructural modification characteristic acute ethanol hepatitis;
2. The histological table was represented by the massive accumulation of lipids characteristic steatosis.
3. In electrono-microscopic aspects were marked the hyperplasia of rough endoplasmic reticulum and hyperplasia of smooth endoplasmic reticulum, degeneration of Golgi apparatus, degeneration of mitochondria and numerous lipids acumulations into cytoplasmic matrice.

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