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FTIR molecular investigation on the concomitant effect of glycine and alcohol induced liver injury

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ABSTRACT

Glycine is known to have a protective role against alcohol induced liver damage. The aim of our study was to evaluate the effect of glycine and alcohol treated albino rats, were analyzed for their lipid content and structural changes using Fourier transform infrared (FTIR) spectral and Light microscopic techniques, respectively. In the administering ethanol (5 g/kg body weight) every day to Wister albino rats for 45 days significantly elevated the lipid levels of liver was found to be increased as compared with the control rats. Simultaneous glycine supplementation (0.6 g/kg body weight) during 45 days of the experiment to rats administered alcohol reduced the lipid levels of tissue. As FTIR measurements are concerned, a vibrational assignment has been for the observed sub-band in the analysis in the C-H stretching region (3000-2800 cm^{-1}) of samples has shown clear changes interpreted on the basis of the structural made in the same samples. Light microscopic examination of ethanol treated rat liver showed inflammatory cell infiltrates and fatty changes, which were reversed on treatment with glycine.

Key words: Glycine; Ethanol; C-H stretching region; Infrared spectra; Light microscopy.

INTRODUCTION

Most risk factors involved in the causation of liver diseases are directly or indirectly due to the disturbances in lipid metabolism [1]. Ethanol is a powerful inducer of hyperlipidemia both in animals and humans beings [2]. Recent data have shown that the beneficial effects of moderate drinking on the risk for cardiac disease [3]. The accumulation of fat in the liver acts as stimulus for the secretion of lipoprotein into the blood stream and development of hyperlipidemia [4].

Glycine is a dietary non-essential aminoacid that can be readily synthesized from common metabolic intermediates in all organisms. Glycine has multiple roles in many reactions such as gluconeogenesis, purine, haem and chlorophyll synthesis and bile acid conjugation [5]. Glycine

lowers the rate of gastric emptying of ethanol resulting in the suppression of its absorption from the gastrointestinal tract [6], in an *in vivo* study of ethanol induced liver injury using the Tsukamoto-French model with a design where alcohol and glycine were given together, glycine lowered ethanol concentration in the stomach and minimized liver damage [7]. Glycine derivatives are also known to decrease considerably the activation of lipid peroxidation in stress, reduce the duration of the alarm stage of stress reaction and limit stress damage to the heart [8]. Glycine is said to activate chloride channels in kupffer cells, which hyperpolarizes the cell membrane and blunts intracellular Ca^{2+} concentration. Similar to its action in the neurons, glycine also decreases the levels of superoxide ions from neutrophils via glycine gated chloride channels [9]. Glycine prevents hepatic cancer and certain melanomas *in vivo* by inhibiting angiogenesis and endothelial cell proliferation [10]. In addition, glycine given orally to schizophrenic patients to facilitate glutamatergic transmission at the level of N-methyl-D-aspartate receptor complex, improved their muscle stiffness and extra pyramidal symptoms [11-12].

Infrared (IR) spectroscopy is a highly sensitive and reproducible analytical technique which is based on the energy of molecular vibrations of several functional groups of organic compounds such as carbonyl, methyl, amide and so on [13]. IR spectroscopy of bio molecules present in entire cells and tissues is a scientific area of increasing importance, with several applications in biophysics, biochemistry and medicine [14]. In recent times, FT-IR technique has provided detailed structural and functional information about biomolecules such as proteins, nucleic acids, lipids and Carbohydrates [15-16]. Recently, Fourier transform infrared absorption (FT-IR) spectroscopy has been extensively employed in this field in order to characterize the structural and chemical/physical properties at molecular level [17].

The enzyme activity can be monitored by either the disappearance of substrate or the appearance of product. FT-IR spectral technique can be used to investigate enzyme kinetics if either the substrates or the products have different spectra. The application of infrared spectroscopy in enzyme assays has been described in the literature for some enzymes such as amidase and $Ca^{(2+)}$-ATPase activities [18].

On histopathological examination, the liver of alcohol and glycine treated rats showed fatty changes of both macro and microvesicular type sinusoidal dilation were observed in all fields. This technique has been widely used for rapid and sensitive determination of macromolecular concentration and conformational changes in biological tissues and membranes [19].

The present work reports a simple and direct spectroscopic method based on FT-IR Technique to study the combined influence of ethanol and glycine on Lipid content in the liver tissue of Albino rats. Furthermore, the present results indicate that almost the same time-course given by morphological changes is fully comparable with those observed by FT-IR spectroscopy.

MATERIALS AND METHODS

Chemicals

Chemicals and reagents used in the present study were of analytical grade and were obtained from Sigma Chemicals Company, Saint Louis, USA and Hi media laboratories, Mumbai, India. Ethanol was obtained from Nellikuppam, Cuddalore District, and South India. Glycine was purchased from S.D. Fine Chemicals Ltd., Mumbai, India.

Animals

Male Wistar Albino rats weighing about 160-180 g were bred in Central Animal House, Rajah Muthiah Medical College, Tamil Nadu, and India. These animals, fed on standard pellet diet (Agro Corporation Private Limited, Bangalore, India) and water *ad libitum*, were used for the present study. The animals were housed in plastic cages under controlled conditions of 12 h light/12 h dark cycle, 50% humidity and at 30° ± 2°C. The animals used in the present study were cared in accordance with the Ethical Committee for Animal Care of Annamalai University and the Indian National Law on Animal Care and use [20]. (Register Number: 166/1999/CPCESA)

Study design

The animals were divided into four groups and treated as follows. Animals continued to receive standard pellet diet and isocaloric glucose from a 40% glucose solution daily by intragastric intubation and served as control group. Animals in group 2 those fed with the standard pellet diet and glycine every day by intragastric intubation for 45 days, group 3 animals received 18% ethanol (5g/kg body weight) solution by intragastric intubation for 45 days and group 4 animals received ethanol and glycine every day by intragastric intubation for 45 days [21].

The animals were monitored closely and average food in take was recorded every day in long-term animals. They were weighed both at the began and end of the experiment. The total experimental duration was 45 days. The animals were fasted overnight and anaesthetized with an intra muscular injection of ketamine hydrochloride (30 mg kg^{-1} body weight) and sacrificed by cervical dislocation at the end of work. The liver samples were collected from control and experimental Albino rats treated with beginning of the section, were initially stored in Phosphate buffer solution with pH 7.4, and then homogenized sample was centrifuged at 3000 rpm for 10 minutes [22]. The membrane-rich sample was collected and oven dried using hot-air oven kept at 120º C for 4 hours to remove the moisture content. The oven dried sample was ground well using Agate mortar and palletized using KBr pellet method. These pellets were irradiated with infrared radiations from the sources of FTIR spectrometer. FTIR spectra for the liver samples of control and experimental animals were recorded in the region the 4000 - 400 cm^{-1} using NICOLET AVATAR – 360 FTIR spectrometer, Nicolet Instrument corporation, Madison, USA made available in CISL, Department of Physics, Annamalai University, Annamalai Nagar, Tamil nadu, India.

Study of change in Lipid content of liver

In order to study the change in Lipid content of liver of 'drug' treated Albino rats, the following semi-quantitative method of analysis was adapted:

The intensity estimates were made using the bands at 2958 cm^{-1}, 2925 cm^{-1}, 2854 cm^{-1} and 1458 cm^{-1}. These bands represent CH_2 (Symmetric and Antisymmetric) and CH_3 (Asymmetric) groups of Lipids. Specific extinction coefficient (K) was calculated for all these three bands using the relation

$$K = DA / m \qquad (cm^2 / gm)$$

Where D was the optical density of the absorption band, A was the area of the pellet (in cm^2) and m was the concentration of the sample in the pellet (in gm).

The K value for these bands present in the FTIR spectra of experimental animals was compared with that of control. Any change in K value, thus obtained, will represent the corresponding change in the chromophoric group and hence, the change in Lipid content of the liver samples.

Sample preparation using Histological processing

Liver of Wistar Albino rats were isolated immediately after euthanasia by cervical dislocation. For paraffin sections liver were fixed overnight in 10% buffered formaldehyde at 4 ºC, washed three times for 2 hrs., in phosphate-buffered saline (PBS) at 4 ºC, and embedded in paraffin. Embedding solvents and times were as follows: 50% Ethanol for 1 hr. at room temperature, 70% Ethanol for 2 hrs., 80% Ethanol for 2 hrs., 96% Ethanol-overnight, 100% Ethanol-three times for 40 min., acetone-for 20 min., xylene-three times for 40 min., paraffin-two times for 1 hr. at 58 ºC, embedding in paraffin at 58 ºC. 8 µm thick sections were cut and mounted on glass slides pretreated with poly-L-lysine. They were deparaffinized in xylene, rehydrated in a descending series of Ethanol and stained by Hematoxylin and Eosin. Pictures were recorded with a Hamamatsu CCD camera and an image analysis system [23].

Using the procedures described above, liver sample was serially sectioned and a minimum of 100 sections per liver sample were stained and studied. Light microscopic studies were made using the Stage microscope available at the Division of Anatomy, Raja Muthiah Medical College, Annamalai University, Annamalainagar, and Tamil Nadu.

RESULTS AND DISCUSSION

Table 1. General band assignment of FT-IR spectrum of liver tissue based on literature

Peak no.	Wavenumber (cm−1)	Definition of the spectral assignment
1	3292	Mainly N-H stretching (amide A) of proteins with the little contribution from O-H stretching of polysaccharides and intermolecular H bonding
2	3015	Olefinic=CH stretching vibration: lipids, cholesterol esters
3	2958	CH_3 asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
4	2925	CH_2 asymmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
5	2873	CH_3 symmetric stretch: mainly proteins, with the little contribution from lipids, carbohydrates, nucleic acids
6	2854	CH^2 symmetric stretch: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
7	1739–1744	Ester C=O stretch: triglycerides, cholesterol esters
8	1654	Amide I (protein C=O stretching): α-helices
9	1542	Amide II (protein N-H bend, C-N stretch): α -helices
10	1458	CH_2 Bending: mainly lipids, with the little contribution from proteins
11	1399	COO^- symmetric stretch: fatty acids and amino acids
12	1236	PO_2-asymmetric stretch: mainly nucleic acids with the little contribution from phospholipids
13	1154	CO-O-C asymmetric stretching: glycogen and nucleic acids
14	1080	PO_2^- symmetric stretching: nucleic acids and phospholipids C-O stretch: glycogen

FTIR absorption measurements

FTIR spectra of the liver of control and treated (Glycine and Ethanol) animals were recorded in the region 4000 – 400 cm^{-1}. The bands were occurred at 3292 cm^{-1}, 2958 cm^{-1}, 2925 cm^{-1}, 2854 cm^{-1}, 2364 cm^{-1}, 1654 cm^{-1}, 1542 cm^{-1}, 1458 cm^{-1}, 1399 cm^{-1}, 1236 cm^{-1} and 1154 cm^{-1}

have been found to be strong and prominently present in all the spectra recorded in the present study.

The present study has been restricted to the C-H stretching region (3000-2800 cm^{-1}) and 1458 cm^{-1} representing the chromophoric groups mainly of lipids and proteins. The Specific extinction coefficient (K) value for bands at 2958 cm^{-1}, 2925 cm^{-1}, 2854 cm^{-1} and 1458 cm^{-1}, characteristic of the chromophoric groups mainly of lipids, were calculated for group 2 and group 4 are slightly increased for lipid levels and group 3 ethanol treated animals significantly higher level of lipid has been detected for long term experimental animals. General band assignments of FT-IR spectrum of liver tissue based on literature [24] are tabulated in (Table 1) and FTIR Spectrum of Liver of Albino rats are shown Fig.1-4.

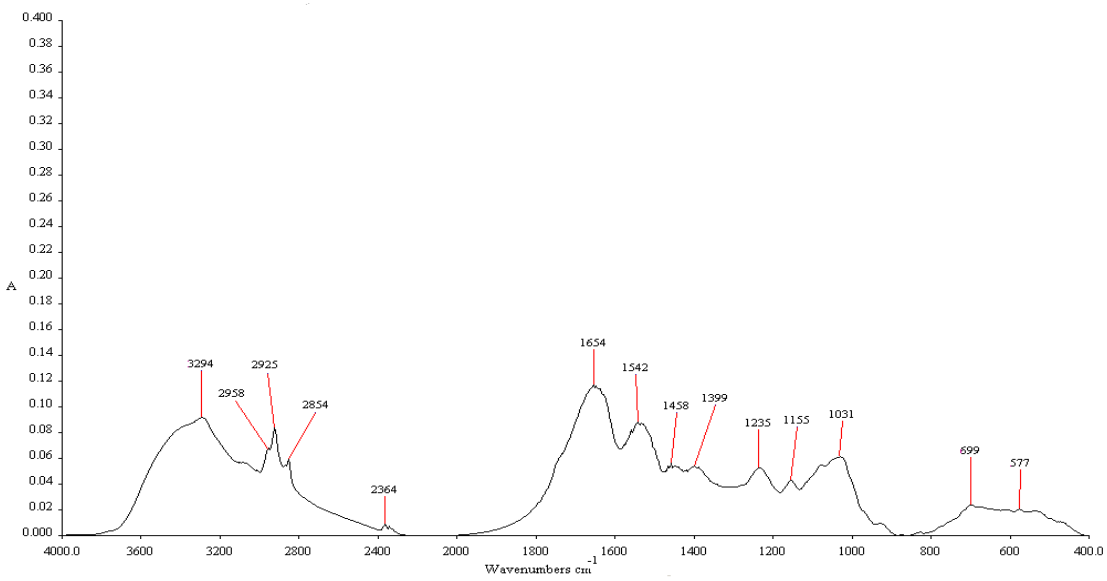

Fig. 1 FTIR Spectrum of Liver of Group 1 (Control) Albino rats

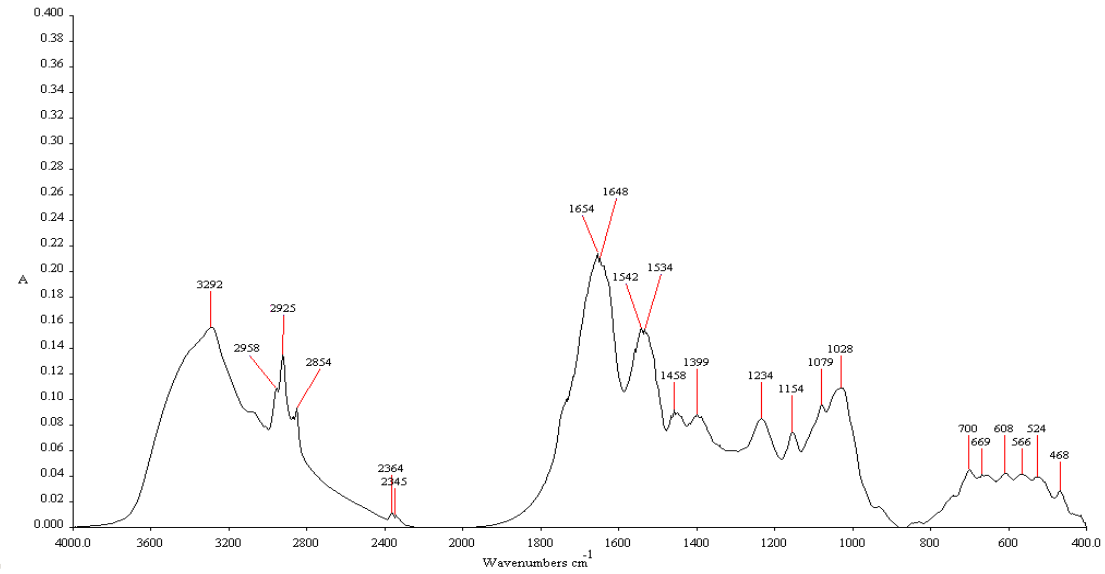

Fig. 2 FTIR Spectrum of Liver of Group 2 (Glycine) Albino rats

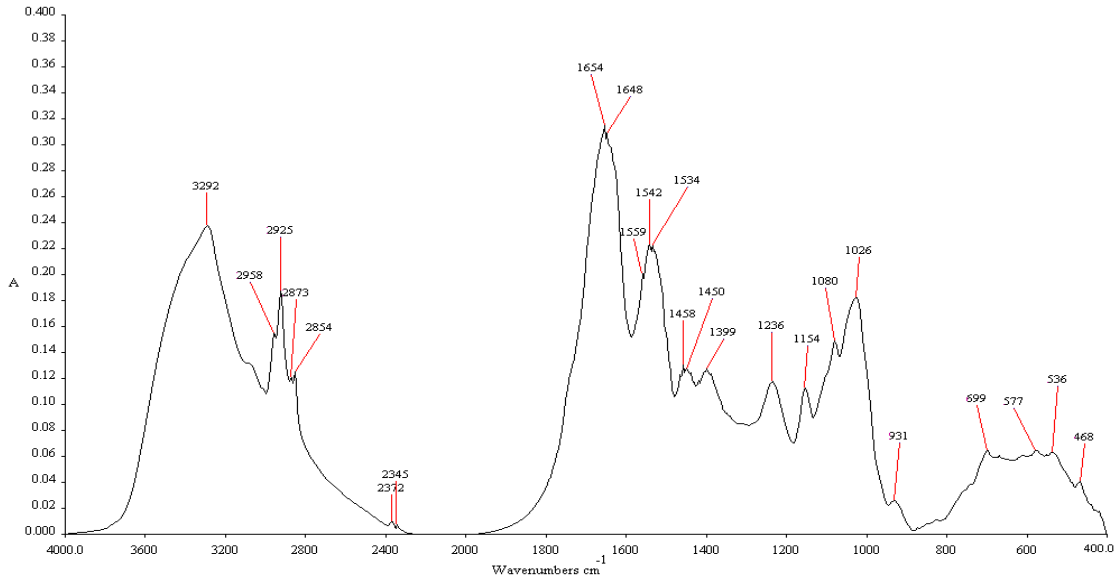

Fig. 3 FTIR Spectrum of Liver of Group 3 (Ethanol) Albino rats

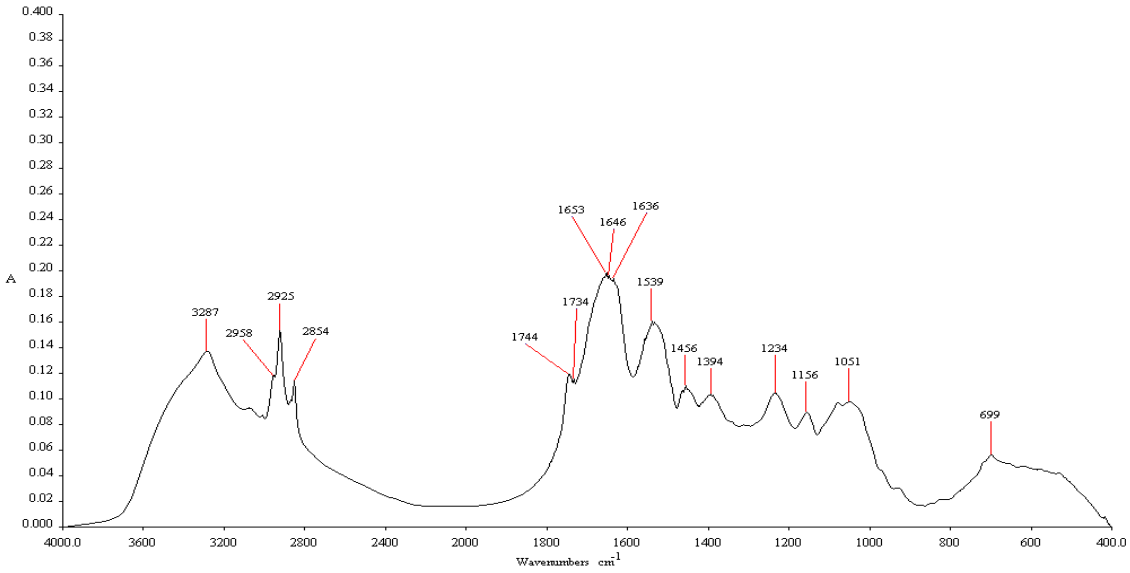

Fig. 4 FTIR Spectrum of Liver of Group 4 (Glycine+Ethanol) Albino rats

Light microscopy measurements

The liver of alcohol treated rat showed fatty changes of both macro and microvesicular type and sinusoidal dilation were observed in all fields (Fig. 7). The liver of alcohol treated rats which received 0.6 g/kg body weight of glycine showed loss of individual hepatocytes by degeneration and the space were the cell had originally been appeared empty, but there was mild macro and microcellular fatty changes and mild portal inflammation (Fig. 8). The liver received 0.6g/kg bodyweight glycine showed only focal areas of fatty changes (Fig. 6). Control liver demonstrated normal liver morphology (Fig. 5).

Alcohol is rich calories and devoid of nutrients, thus contributing to accumulation of fat in the liver. On the other hand, alcohol is known to reduce the absorption of other foodstuff and nutrients from intestine [25]. It is known that the dominant lipid bands in the 3030–2800 cm^{-1} region originate from the C-H stretching vibrations of the fatty acyl chains of membrane lipids [26]. The intensity or area of IR absorptions arising from a particular species is

directly proportional to the concentration of that species [27]. As the calculated Specific extinction coefficient (K) is based on the IR band intensity, any change in 'K' value can be taken to be directly proportional to the concentration of the concerned species. From Table 2 an increase in the 'K' values has been noticed for the bands at 2958 cm^{-1}, 2926 cm^{-1}, 2854 cm^{-1} and 1458 cm^{-1} which were assigned as olefinic = CH band in the average spectrum of the rat testis in the control and experimental groups. The intensity of the olefinic = CH band can be used as an index of relative concentration of double bonds in the lipid structure of unsaturated lipids [28]. These double bonds mainly originate from lipid peroxidative end products, such as malondialdehyde. Consequently, it is possible to deduce from the present study that there is an increased peroxidative process in the liver of treated animals [29]. In my present studies, the significant increase in the intensities of the CH_2 symmetric and asymmetric stretching bands in all the investigated regions of the group 3 corresponds to an increase in the lipid content are tabulated in (Table 2).

Table 2. Effect of control and treated on lipid in rat liver – using FTIR study

Infrared Band (cm^{-1})	Sample	Specific extinction coefficient (K) (cm^2/gm)	% variation with respect to S_1
2958	S_1	42.1813	
	S_2	46.2272	+09.59
	S_3	83.5274	+98.02
	S_4	49.5231	+17.41
2925	S_1	56.3247	
	S_2	60.2174	+09.22
	S_3	98.4524	+74.79
	S_4	62.1531	+10.35
2854	S_1	36.2841	
	S_2	39.6278	+09.22
	S_3	77.6543	+114.02
	S_4	40.1274	+10.59
1458	S_1	35.9247	
	S_2	39.1271	+08.91
	S_3	76.3457	+112.52
	S_4	39.9243	+11.13

+ indicates an increment

$S_{1 - Control}$ $S_{3 - treated}$ *for ethanol*

S_2 *– treated for glycine* S_4 *– treated for glycine+ethanol*

One of these conditions, relevant to alcohol-induced liver injury, is the presence of increased amounts of lipid in the liver [30]. The height and area of asymmetric and symmetric C-H stretching bands have been found to be proportional to the amount of the lipids present in the epidermis. Therefore, the increase in the 'K' values calculated in the present study can be attributed to epidermal lipid accumulation [31].

The findings of the present study indicating an increase in the lipid level for the long-term treated Albino rat liver are significant in this aspect since they reveal that lipid metabolism is altered at an early onset of liver. This increase in the lipid content might lead to cardiomyopathy in diabetes, as suggested by others [32].

Liver is the main target organ for ethanol. Alcohol administration produces a spectrum of histological abnormalities in the liver [33]. Fatty change is commonly seen in alcoholic liver disease. Alcohol administered rats showed lipid accumulation in large (macrovesicular) and small (microvesicular) droplets within hepatocytes. They can simulate feathery degeneration of hepatocytes, a pattern seen when a liver cell retains both bile salts and water. Feathery degeneration is the swelling of hepatocytes with increased pale cytoplasm and non-specifically, leads to lytic necrosis and replacement by inflammatory cells. Some of these hepatocytes will die or become apoptotic bodies which in turn could be taken by hepatocytes or Kupffer cells. Kupffer cells become much more prominent when they contain phagocytosed material. They were enlarged and proliferate when liver is injured (Kupffer cell hyperplasia). Chronic liver disease leads to fibrosis which leads to derangement of the architecture, portal hypertension and may produce such an irreversible rearrangement of the circulation. Congestion is a passive process and occurs as the result of impaired venous drainage [34]. Histopathological changes of liver of experimental rats were shown in Fig. 5 to 8.

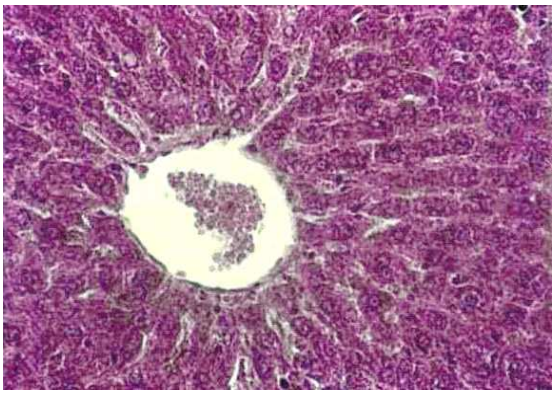

Fig. 5 Control Normal Histology-Central vein

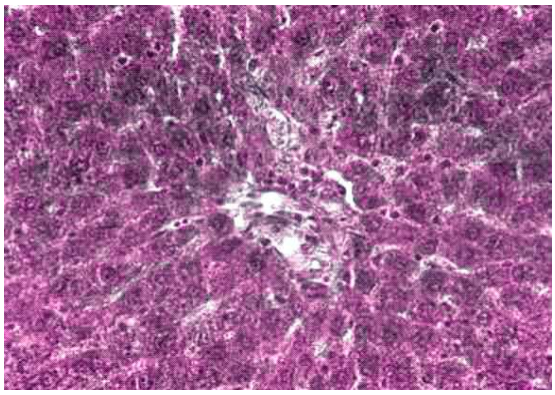

Fig.6 Glycine Normal Histology

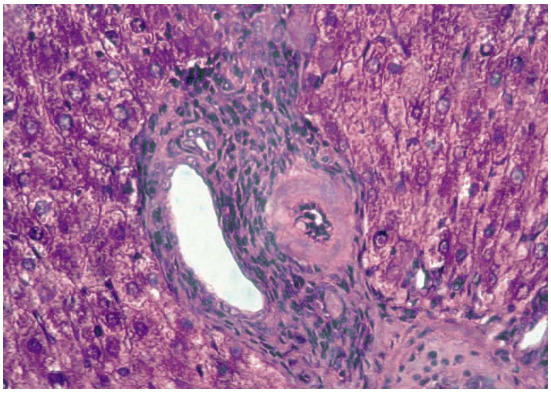

Fig.7 Ethanol Micro and Macro cellular fatty changes, feathery degeneration and periportal fibrosis

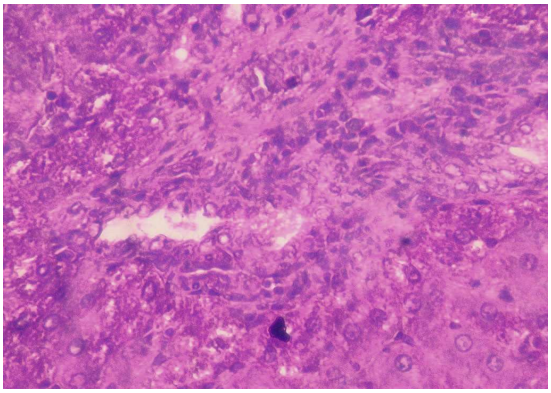

Fig.8 Ethanol + Glycine mild micro and macro cellular fatty changes and mild portal inflammation

Thus the histopathological examination of the liver of alcohol administered animals show micro and macro cellular fatty changes, feathery degeneration, bile stasis, Kupffer cell hyperplasia, portal inflammation, periportal fibrosis and vascular congestion. These changes were predominant in the centrilobular region having reduced oxygen perfusion. Hepatic damage may be partially attributed to cytochrome-P450 dependent enzyme activities in liver that tends to be

present in greatest concentration near the central vein and lower near the peripheral sites [35]. On treatment with glycine, the liver showed normal histology with mild congestion of central vein.

CONCLUSION

Our work was addressed to characterize, by the employment of coupled methods, the damage and regeneration caused by ethanol administration in rat liver. With the support of the obtained structural results, it is clear that the FT-IR absorption technique provides reliable, reproducible spectra with can be used to unambiguously distinguish normal from injured specimens.

Finally, we want to remark that the functional methods and morphological data together with spectroscopic techniques are essential for studying the complex problem of the liver damages caused by different foreign agents.

An extension of the present work is in progress in our laboratories in order to study the C-H bands evolution in the time passing to complete the regeneration and support the morphological results which claim that the restitutio ad integrum occurs after 45 days from Glycine and ethanol administration.

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