

Study to Evaluate Hepatotoxic Characterizations Induced Through Ethanol in Albino Rats *Mus Musculus*

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Abstract

Objective: To determine structural and functional hepatic characterizations induced through the ethanol in albino rats *mus musculus*.

Study Design: This study was an experimental Randomized Control Trial (RCT).

Place and Duration: The study was conducted at the experimental research laboratory of Government College University Lahore with collaboration of College of Medicine Al-Kharj University and Obeid Specialized Hospital, Riyadh, Saudi Arabia from January 2010 to December 2010.

Material and Methods: Hundred male albino rats of 6 – 8 weeks old, weighing 150 – 200 gm each were divided into two groups of fifty each. Group A served as control and was given normal rat diet; Group B was given ethanol at a dose of 0.6 ml (0.5 gm) / 100 gm / day for 8 weeks. At the end of the experiment, blood was drawn from each animal by cardiac puncture for liver function tests. Each animal was then sacrificed under chloroform anaesthesia and its liver was removed.

Results: The mean values of Serum alanin-amino-transferase (ALT) and gamma glutamyl transferase (GGT) in group A were 28.17 ± 7.13 and 28.33 ± 3.05 respectively; where as in group B, the mean values of these enzymes were 84.33 ± 10.89 and 80.33 ± 4.37 U/L respectively, Students “t test showed statistically significant increase in the mean values of ALT and GGT in group B as compared with those in group A, p value being < 0.05 . Liver was normal in appearance in all animals. The mean weight and volume of the liver in group A were 10.90 ± 0.39 gm and 10.33 ± 0.42 ml respectively; where as, in group B, these were 13.25 ± 0.50 gm and 13.23 ± 0.38 ml respectively. Students “t” test showed statistically significant difference (p value being < 0.05) for weight and volume of the liver of group B when compared with those in group A. Liver preparations for histological study were examined under the light microscope. Group A showed normal liver histology, the mean size of hepatocytes and their nuclei was 21.25 ± 0.38 and 7.52 ± 0.12 μm respectively. In – group B, the mean size of hepatocytes and

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their nuclei was 26.24 ± 0.54 and 7.53 ± 0.12 μm respectively; the hepatocytes contained large number of cytoplasmic vacuoles, pyknotic nuclei were also observed and portal areas showed lymphocytes infiltration;. Students "t" test showed statistically significant difference in the mean value of the size of hepatocytes; Chi-square test also showed statistically significant difference in the percentage of hepatocytes containing cytoplasmic vacuoles, pyknotic nuclei and percentage of portal areas showing lymphocytic infiltration in the of the liver of the animals in group B, when compared with those in group A (p value being < 0.05).

Conclusion: Ethanol is hepatotoxic in albino rats as evident from the functional disorder and structural changes in the live.

Key Words: Ethanol, hepatotoxicity Pyknotic nuclei, Hepatocytes.

Introduction

Ethanol induces a number of deleterious metabolic effects in the liver such as increase production of lactate, fatty and uric acids; its excessive use for a long time leads to development of alcoholic liver disease. The three most widely recognized forms of alcoholic liver diseases are fatty liver / steatosis, alcoholic hepatitis and liver cirrhosis. At least 80% of heavy drinkers have been reported to develop steatosis, 10% – 35% alcoholic hepatitis, and approximately 10% liver cirrhosis.^{1,2}

Steatosis is the early stage of alcoholic liver damage, which is reversible with abstinence from alcohol (ethanol) but progresses to cirrhosis, if excessive use of alcohol continues. The fat may be in the form of micro vesicular (small droplet) or macro vesicular (large droplet). Macroscopically, the fatty liver is a large, soft organ, which is yellow and greasy. Fatty liver seems to be a prerequisite to the development of alcoholic hepatitis and becomes more vulnerable to getting inflamed.³ In alcoholic hepatitis, there is swelling of hepatocytes due to fat and water accumulation. The nucleus is small and hypochromatic and there is inflammation and necrosis of hepatocytes. Lymphocytes and macrophages enter portal tracts and spill into the hepatic parenchyma. Alcoholic hepatitis is usually associated with proliferation of sinusoidal stellate cells and portal tract fibroblast, giving rise to sinusoidal and peri-venular fibrosis.^{3,4} The final and irreversible form of alcoholic liver disease is alcoholic cirrhosis, in which collagen deposition occurs in the space of Disse, and around central veins; this is severe form of alcoho-

lic liver injury and is usually of the micro nodular type, which prevents blood from travelling freely through liver, leading to portal hypertension and its complication.⁵

Recent studies in animal models suggest that liver injury in chronic alcoholics is produced on account of oxidative stress. Progress of disease involves continuing injury to hepatocytes, fibrosis, and impaired liver functions. Various experimental studies described that ethanol caused accumulation of reactive oxygen species like super oxide, hydroxyl radical, and hydrogen peroxide in hepatocytes; these oxidize the reduced glutathione, which in turn leads to lipid per oxidation of cellular membranes, and oxidation of protein and DNA, resulting in hepatocytes injury.⁶

It had been reported earlier that ethanol increased weight and volume of the liver,⁷ produced fat vacuoles in hepatocytes.⁸ Apoptosis had been observed in the liver after treatment with ethanol⁹ and changes in liver function tests upon ethanol consumption had also been extensively studied in the past.¹⁰ The present study was designed to make comprehensive investigations on hepatotoxic effects of ethanol, which included functional derangement of the liver and changes in its structure – a correlative study, it is hoped that it might serve as warning to those, who have the tendency to indulge in drinking.

Material and Methods

This study was an experimental Randomized Control Trial (RCT) conducted at the Experimental Research Laboratory of Government College University Lahore. One hundred male albino rats 6-8 week old, weighing 150 – 200 gm each were procured from National Institute of Health, Islamabad. They were kept under controlled temperature (23 – 25°C), humidity (60%) and light and dark cycles of 12 hours each and allowed to acclimatize for one week. The animals were fed on standard rat diet and water ad libitum, and were weighed at the start of experiment. The animals were randomly divided into two groups, having fifty rats each. Group A served as control and were given 2 ml / 100 gm / day distilled water by mouth, in addition to water ad libitum. Group B served as experimental and was given 2 ml / 100 gm body weight per day of 30% v/v of aqueous solution of ethanol containing 0.6 ml (0.5 gm) of ethanol by mouth for 8 week. The body weight of each animal was recorded twice weekly and at the end of the experimental period.

At the end of experiment, each animal was taken out of cage and was euthanized under chloroform before 6ml of blood was taken in 10 ml disposable syringe by cardiac puncture. The blood sample was allowed to stand for one hour and centrifuged at a speed of 3000 rpm for 10 minutes. The clear serum was collected with the help of a clean dropper in sterilized disposable plastic tubes. These plastic tubes were then placed in freezer and stored at -20°C for testing on a later date; the tubes were properly labelled. Serum alanin-aminotransferase (ALT) and gamma glutamyl transferase (GGT) levels were measured by using commercially available kits of “Human Company”.

Each animal was then sacrificed. The liver of each animal was removed and examined for gross changes; 2 mm² pieces were taken from different sites of liver; these were fixed in 10% formaldehyde for 48 hours and then processed in a usual way by dehydrating with ascending grades of ethanol, clearing in Xylene and infiltrating with molten paraffin of melting point 56°C. Paraffin blocks were made using base moulds; these were then placed in the refrigerator for fifteen minutes before sectioning. Five micron thick sections were obtained using Leica RM 2125 rotary microtome; these were then floated on hot water bath at 45°C temperature for stretching the folds; the sections were picked up on top of albumenized glass slide and stained with H&E and PAS stains,¹¹ the slides were then examined under light microscope using different magnifications.

The data was analyzed using SPSS version 15.0. Mean ± SE was given for quantitative variables. Frequencies and percentages were given for qualitative variables. Two independent sample t test was applied to observe group mean different between two groups.

Fischer exact test was applied to observe association between qualitative variables. Differences between groups were considered to be statistically significant, if p value was < 0.05.

Observations and Results

All animals of group A were healthy and active; however, the animals of group B suffered slight degree of drowsiness. In group A, the mean body weight of the animals at the start and at the end of experiment was 68.33 ± 4.40 and 208.16 ± 5.84 gm respectively; whereas, in group B, the mean body weight of the animals at the start and at the end of experiment was 165.66 ± 4.46 and 182.66 ± 3.5 gm respectively. Students ‘t’ test did not show statistically significant difference in the mean body weight of animals between groups A and B at the start of experiment (p value being > 0.05), however there was statistically significant difference in the mean body weight of animals between groups A and B at the end of experimental period, p value being < 0.05 (Table 1).

In – group A, the mean values of serum alanin amino-transferase (ALT) and serum gamma glutamyl transferase (GGT) were 28.17 ± 7.13 and 28.33 ± 3.05U/L respectively; whereas in – group B, the mean values of these enzymes were 84.33 ± 10.89 and 80.33 ± 4.37 U/L respectively. Students ‘t’ test showed that there was statistically significant increase in the mean values of serum alanin amino transferase (ALT) and gamma glutamyl transferase (GGT) in group B ,when compared to those in group A, p value being < 0.05 (Table 2).

Table 1: Mean body weight of animals in gm at the start and at the end of experiment.

Body weight of animals	Group A (n = 6) Mean ± S.E	Group B (n = 6) Mean ± S.E	p. value
At the start	168.33 ± 4.4	165.66 ± 4.46	0.3211
At the end	208.16 ± 5.84	182.66 ± 3.5	< 0.001

p value (<0.05) is statistically significant.

Table 2: Mean value of serum alanin amino transferase (ALT) and serum Gamma glutamyl amino transferase (GGT) in U/L.

Serum Enzymes	Group A (n = 6) Mean ± S.E	Group B (n = 6) Mean ± S.E	p. value
ALT	28.17 ± 7.13	84.33 ± 10.89	< 0.001
GGT	28.33 ± 3.05	80.33 ± 4.37	< 0.001

Liver of group A was reddish brown with smooth and shiny surface; it was invested by thin glistening capsule which was not adherent to adjoining organs; it possessed four, well demarcated lobes and showed no obvious gross abnormalities. In – group B, liver of all animals was normal in shape and colour and, was comparable to that in group A. The mean weight and mean volume of liver in group A were 10.78 ± 0.39 gm and 10.33 ± 0.42 ml respectively; whereas, those in group B were 13.23 ± 0.50 gm and 13.23 ± 0.38 ml respectively. Students' t' test showed that there were statistically significant differences in mean weight and volume of the liver between groups A and B, p value being < 0.05 .

Histological Examination of Liver

The histological sections of liver of group A, showed hepatic lobules with central vein in the center (Fig. 1). The laminae of hepatocytes appeared as cords of polyhedral cells, radiating to the periphery of the lobule; hepatic sinusoids were enclosed between them, and were lined by discontinuous endothelial cells with flattened nuclei; the hepatic sinusoids were observed to anastomose irregularly with each other and opened into the lumen of central vein, which was lined by flattened cells and surrounded by thin layer of fibrous tissue (Fig. 1). The hepatic cells were polyhedral in form and had sharply defined boundaries; the cytoplasm of some of these cells contained small vacuoles of a variable size and shape, presumably indicating glycogen

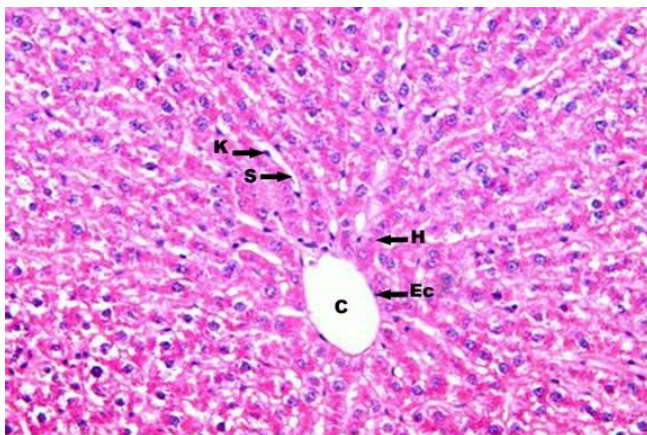


Fig. 1: Photomicrograph of a section from the liver of group A, showing central vein (C) lined by endothelial cells (Ec) surrounded by radiating cords of hepatocytes (H) enclosing hepatic sinusoids (S), lined with Kupffer cells (K). H and E stain. X200.

or fatty content of the cytoplasm that had been removed during tissue processing. Each hepatocyte contained a vesicular nucleus with 1 – 2 nucleoli in it. Some hepatocytes were binucleated (Fig. 2). The mean size of hepatocytes and their nuclei was 21.24 ± 0.38 and 7.51 ± 0.12 μm respectively. The mean diameter of central vein in group A was 78.50 ± 0.99 μm . Portal area, comprising branch of portal vein, hepatic artery and bile duct was also observed. The branch of portal vein had wide lumen, lined by endothelial cells; the branch of hepatic artery had narrow lumen and thick wall as compared to branch of portal vein. The bile duct was lined by low cuboidal epithelium (Fig. 2).

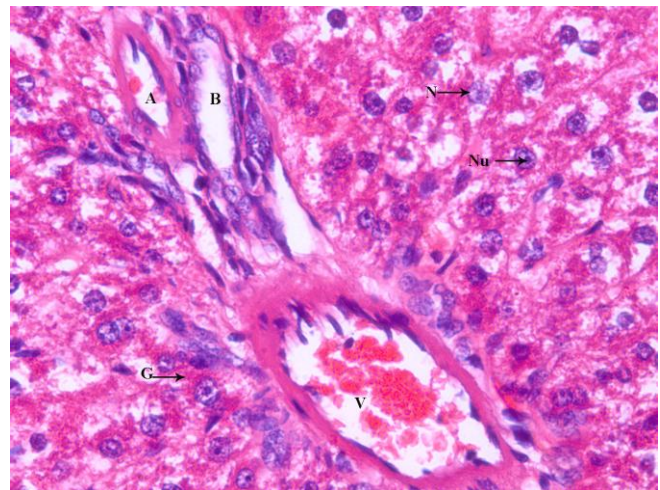


Fig. 2: Photomicrograph of a section from the liver of group A, showing portal area comprising portal vein (V), hepatic artery (A) and bile duct (B). The surrounding cells contain glycogen granules (G); their nuclei are central (N) with one or two nucleoli (Nu), PAS stain. X 400.

In histological sections from group B, the hepatocytes were arranged in the usual form of hepatic cords radiating from central vein toward the periphery of the hepatic lobules, and were comparable to those in group A (Fig. 3). Size of the hepatocytes in group B, was larger than that of hepatocytes from group A; the cytoplasm of the cells, however, contained large number of micro and macro vacuoles involving whole of the hepatic lobule. Nuclei of hepatocytes of this group appeared vesicular with a distinct nuclear envelope containing one or two prominent nucleoli and scattered chromatin. Some hepatocytes contained two nuclei. Pyknotic nuclei were also observed (Fig. 4). The mean

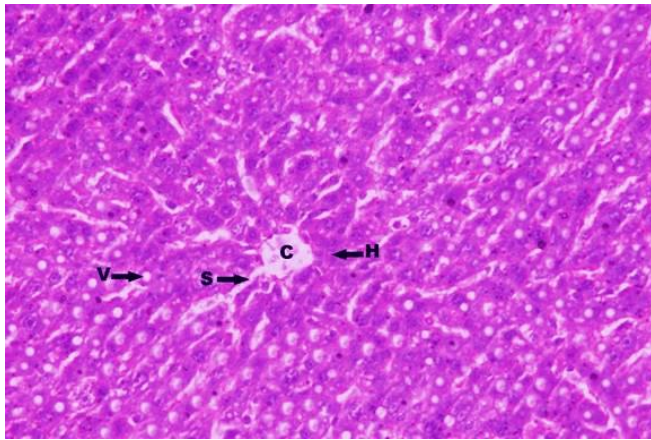


Fig. 3: Photomicrograph of a section from the liver of group B, showing central vein (C), continuing with hepatic sinusoids (S); surrounded by cords of hepatocytes (H) that contain cytoplasmic vacuoles (V). H and E stain. X200.

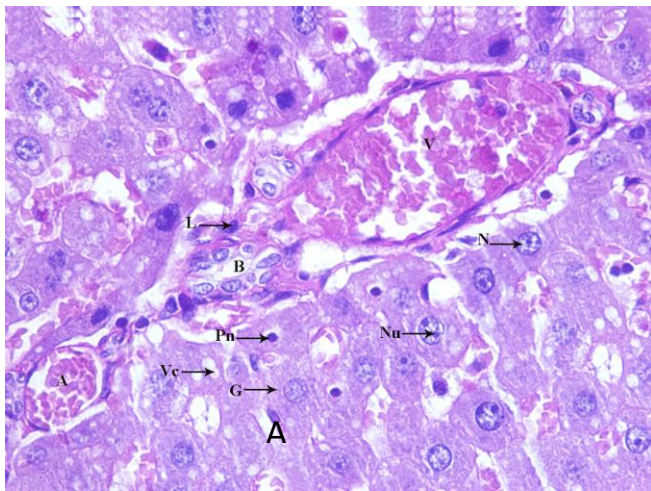


Fig. 4: Photomicrograph of a section from the liver of group B, showing portal area comprising portal vein (V), hepatic artery (A) and bile duct (B); the surrounding cells contain glycogen granules (G) and cytoplasmic vacuoles (Vc), nuclei (N) contain one or two nucleoli (Nu); there is lymphocyte infiltration (L) in portal area, pyknotic nuclei (Pn) are also observed. PAS stain. X400.

size of hepatocytes and their nuclei was 26.23 ± 0.54 and $7.51 \pm 0.12 \mu\text{m}$ respectively. The mean diameter of central vein was $79.16 \pm 1.35 \mu\text{m}$. The portal area showed lymphocytes infiltration; the portal vein and hepatic artery contained erythrocytes and bile duct was lined with cuboidal cells and appeared normal (Fig. 4).

Students ‘t’ test showed statistically significant increase in the mean size of hepatocytes in group B when compared with group A (p value being < 0.05); there was, however, no statistically significant difference in mean size of central vein, when those from group B were compared to group A, p value being >0.05 , (Table 3).

Fisher exact test showed statistically significant difference between groups A and B regarding percentage of hepatocytes containing cytoplasmic vacuoles (p value = 0.002) whereas difference in pyknotic nuclei between groups A and B was statistically significant (p value = 0.006). Difference in percentage of portal area showing lymphocytic infiltration, when compared between the groups A and B was also statistically significant (p value = 0.0056).

Discussion

In the current investigations we observed that ethanol produced hepatotoxic effects in rats, as manifested by significant increase in the serum levels of alanine amino-transferase (ALT) and gamma glutamyl transaminase (GGT) in group B, when compared to those in group A (p value being < 0.05); this was presumably due to production of reactive oxygen species, inducing protein oxidation and lipid per oxidation which resulted in hepatocyte injury. These observations were comparable to those reported by Enomoto,¹⁰ who observed the effect of Pioglitazone in prevention of ethanol induced liver injury in rats.

There was significant increase in weight and volume of the liver of group B, when compared with those of group A; this may be due to accumulation of fats, water and increased size of hepatocytes. Saravanan⁷

Table 3: Mean size of hepatocytes, their Nuclei, and central veins in microns (μm).

Components of lobules	Group A (n = 6) Mean \pm S.E	Group B (n = 6) Mean \pm S.E	p.value
Hepatocytes	21.24 ± 0.38	26.23 ± 0.54	< 0.001
Central vein	78.5 ± 0.99	79.16 ± 1.35	> 0.03570

p value (<0.05) is statistically significant.

studied antioxidant effect of *Hemidesmus indicus* on ethanol induced hepatotoxicity in rats and reported comparable results. Our observations showed statistically significant increase in size of hepatocytes, number of micro and macro cytoplasmic vacuoles in group B when compared to those of group A (p value being < 0.05). This was presumably due to accumulation of fats, protein and water; our results corroborate those reported earlier by Ronis⁸ who reported that ingestion of ethanol along with low carbohydrate diet in rats produced vacuoles and inflammatory changes in the liver. In group B, the nuclei of hepatocytes were larger and vesicular with dispersed chromatin materials; there was, however, no statistically significant change in size of nuclei of hepatocytes of group B, when compared to those of group A (p value being > 0.05). There was no statistically significant change in the diameter of central vein in group B as compared to group A (p value being > 0.05). Pyknotic nuclei were observed in group B; the difference in number of pyknotic nuclei of the group B was statistically significant, when compared with those in group A (p value being <0.05). These observations were in accord with those of Manuela⁹ who observed the effects of ethanol on hepatocyte cultures and reported that ethanol induced apoptosis characterized by Pyknotic nuclei which appeared as dark and irreversible condensation of chromatin. Liver preparation from rats in group B showed lymphocytes infiltration around the bile duct called periportal inflammation; this finding was also reported by Yin.¹² who observed peri portal lymphocyte infiltration in ethanol induced hepatotoxicity in rats. Evidence of liver cirrhosis was not observed in animal of our experimental group B which possibly would have developed eventually, if the animals were given ethanol for a longer period.

Conclusion

Ethanol treated albino rats showed a fair degree of derangement of liver functions, associated with con-

comitant changes in the histological structure of the organ.

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