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The Effect of Curcumin on Experimental Non- alcoholic Fatty Liver Disease in Rat Models,"Histochemical and Immunohistochemical Studies"

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Abstract

Introduction: Nonalcoholic fatty liver disease is the most common and emerging form of chronic liver disease worldwide. It includes a wide spectrum of liver diseases ranging from simple fatty liver to steatohepatitis, which may progress to cirrhosis, liver cancer, and liver mortality.Curcumin was demonstrated to limit activation of the master inflammatory factors, nuclear factor-Kappa β [NF-k β] and to block oxidative injury. Objective: to investigate the effect of curcumin on NAFLD by histochemical detection of catalase activity and immunohistochemical determination of NF-k β expression in the liver tissue.

Materials and Methods: Sixty Wistar male rats were divided into four groups: Group I: Control group received rat chew diet for 12 weeks. Group II: Fatty liver group, animals received high-fat diet for 12 weeks. Group III: Fatty liver group injected intraperitoneally [IP] with 1 ml/kg body weight dimethyl sulfoxide [DMSO] every other day for 8 weeks. Group IV: Fatty liver group injected with 50 mg/kg body weight, curcumin dissolved in DMSO, IP every other day for 8 weeks. Animals were sacrificed at the end of the experiment. Liver specimens were stained with hematoxylin and eosin (H&E)for histopathological study. Diaminobenzidine(DAB) method was used for for histochemical detection of catalase activity. The grades of catalase stainability were detected. NF-k β expression in liver tissues was determined using NF kappa B/P65 Rabbit polyoclonal antibody and UltraVision detection System. The percentage of positively stained cells was recorded.

Results: Histopathology of NAFLD group revealed marked hepatic degeneration,while curcumin treatment showed normal structure. Catalase activity decreased after NAFLD induction and increased after treatment by curcumin. NF-k β expression was higher in NAFLD group and DMSO groups compared to

control group ($p<0.05$). Curcumin treatment in group IV significantly decreased expression of NF-k β compared to NAFLD and DMSO groups .

Conclusions: Curcumin has antioxidant effect by increasing activity of catalase and has reduced inflammation through decreasing NF-k β activity in the liver tissue.

Keywords: Non-alcoholic fatty liver, Curcumin, Nuclear factor kappa- β , Catalase, Liver tissue

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a pandemic liver disease from the twenty-first century. It has been estimated that one billion individuals worldwide have NAFLD [1]. The term NAFLD refers to a spectrum of hepatic disorders, ranging from simple fatty liver (NAFL, Nonalcoholic fatty liver) in which no inflammatory changes are seen except for macrovascular or microvascular steatosis to non-alcoholic steatohepatitis (NASH) , which is characterized by an inflammatory reaction with hepatocyte injury, such as balloonic degeneration and necroapoptosis with or without fibrosis [2].

Hyperinsulinemia leads to steatosis. After the development of steatosis, the adipokine/cytokine imbalance, bacterial toxins in the intestine and oxidative stress leads to activation of stellate cells and Kupffer cells which eventually leads to liver injury. Nuclear factor-Kappa β (NF-k β) is a transcription factor present in an inactive form in the cytoplasm, this inactive form has protein kappa β alpha [Ik β a] subunit. On activation, Ik β a undergoes phosphorylation and ubiquitination-dependent degradation, resulting in gene transcription [3]. There were previous experiments confirmed that in NAFLD rat liver tissue, NF-k β expression was significantly enhanced, compared to normal rats [4]. These studies concluded that NF-k β signaling pathway has a role in the pathological process of NAFLD.

Interest in antioxidants and antioxidant therapy has been growing during the last decade. Antioxidants are generally considered to have the capability to protect people from harmful effects of reactive oxygen and nitrogen species, including free radicals, when these are present in excessive amounts [5].

Curcumin, extracted from Curcuma longa herb, is known to have anti-oncogenic protective effects due to its anti-oxidative characteristics [6, 7, 8]. It has been revealed to limit the activity of inflammatory transcription factors, reduce oxidative stress, and to suppress pro-fibrogenic cytokines and connective tissue growth factors in hepatic stellate cells [HSCs] [9, 10]. Moreover, curcumin has been shown to limit multiple signaling pathways and to modify proteins and gene product for cell endurance and proliferation [11].

The present work aims to investigate the effect of curcumin on NAFLD by histochemical detection of catalase activity and immunohistochemical determination of NF-k β expression in the liver tissues.

2. Aim of the work

Being of low cost and of negligible toxicity, it is important to investigate the role of curcumin in experimental NAFLD as a pre-clinical research. The present work aims to investigate the effect of curcumin on experimental NAFLD by histochemical study of catalase activity and immuohistochemical study of NF-k β expression in the liver tissue.

3. Materials and Methods

All procedures were done according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and after approval of the local Animal Ethics Committee. This study was conducted on 60 Wistar male rats (devided on 4 equal groups) weighing approximately 100-120 grams obtained from Animal House of Medical Research Institute, Alexandria University. Animals were housed 5 per cage with food and water ad libitum, on 12:12-h light-dark cycle at 23 ± 1 ° C. Rats were divided equally into four groups.

Group I: "The control group" which received standard rat chew diet [23 % of protein, 49 % of

carbohydrate, 4 % of total fat, 5 % of fiber, 7 % of ash, and 6 % of vitamins] for 12 weeks.

Group II:"The fatty liver group" animals received high-fat diet [12]20% protein, 20% fat, 48% carbohydrate, and 4% fiber] for 12 weeks.

Group III: Fatty liver group injected intra-pretoneally (IP) with 1 ml/kg body weight dimethyl sulfoxide [DMSO][Sigma,Aldrich]every other day for 8 weeks.

Group IV: Fatty liver group injected IP with 50 mg/kg body weight, curcumin [Sigma,Aldrich] dissolved in DMSO, every other day for 8 weeks.

Animals were sacrificed at the end of the experiment. Blood and liver samples were collected.

Histological study: Liver specimen were preserved in paraffin, sectioned and stained with hematoxylin and eosin (H&E)for histopathological examination.

Histochemical study: Liver tissues were frozed in -30 ˚C and sectioned for histochemical study of catalase using diaminobenzidine (DAB) method. Grades of catalase stainability, which reflect intensity of catalase activity, were graded semiquantitatively as follows: Intense reaction: ++++, marked reaction: +++, moderate reaction : ++, weak reaction : +, and negative reaction: 0 [13]

Immuohistochemical study: NF-k β expression by Avidin-Biotin Complex (ABC) method using NF kappa B/P65 Rabbit polyoclonal antibody and UltraVision detection System (Anti-polyvalent , HRP/ DAB Plus) (Thermo Fisher Scientific Fremont, CA 94538, USA)[14]. The percentage of positively stained cells was determined by counting a minimum of 100 cells, and the average was recorded, and scored as follows: (0)=absent staining, (1)=weak, <10% of cells were positive; (2)=moderate, 10-50% of cells were positive or (3) = strong, >50% of cells were positive.

Statistical analysis was performed with Kruskal-Wallis test to compare grading score between the four groups and pairwise comparison between each two groups.

4. Results

4.1 Histological Results

Group I [Normal control group]: Normal control liver tissue sections stained with H&E stains show normal hepatic lobules. Each lobule showed anastomosing plates of hepatocytes radiated from the central vein toward the periphery of the lobule [Figure 1.a].

Group II [NAFL group]: This group showed evidence of marked hepatic degeneration with the presence of micro and macrovesicular steatosis. We also noticed the congested portal tract and vaculated cytoplasm [Figure 1.b].

Group III [DMSO group]: After 8 weeks: regeneration of the hepatocytes with the presence of minimal inflammation and congestion. We also noticed microvesicular fat droplets and increased number of Kupffer cells [Figure 1.c].

Group IV [curcumin group]: After 8 weeks: liver sections showed more or less the appearance of normal structure with the presence of radiated lobules and reappearance of binucleated nuclei but there were also few necrotic cells and few degenerated cells [Figure 1.d].

4.2 Histochemical results

Catalase [Diaminobenzidine(DAB) method] arbitary stainability is shown in Figure 2.

Group I [control]: Catalase activity was illustrated as moderate granular reaction distributed in the cytoplasm. [Figure 3.a]

Group II [NAFL]: Rat liver sections of this group showed weak catalase activity while there was some intense catalase activity in the cytoplasm of some hepatocytes.[Figure 3.b]

Group III [DMSO]: Marked activity of catalase was noticed after 8 weeks[Figure 3.c].

Group IV [curcumin]: After 8 weeks marked catalase activity was shown [Figure 3.d].

4.3 Immunohistochemical Results

Immunohistochemical staining of nuclear factor kappa B [NF-kB] expression scores in the four groups with their statistical comparisons are illustrated in Figure 4.

Group I [control]: NF-kβ expressed in pericentral area, perisinusoidal area and in most nuclei of Kupffer cells as brown granules [Figure 5.a].

Group II [NAFL]: In this group we noticed that there was down regulation of IKB gene expression especially in cytoplasm which may partly explain increased activity of NF-kβ. We also noticed weak reactivity in pericentral area of rat

liver section and periportal area [Figure 5.b] with significance [p<0.05] compared to control group.

Group III [DMSO]: This group showed that the immune reactivity for p65 subunit of NF-kβ was localized exclusively to the cytoplasm of hepatocytes. This cytoplasmic expression was increased [Figure 5.c] after [8 weeks] [p<0.001].

Group IV [curcumin treated group]: The liver has been shown to limit activation of NF-kβ by directly bounding and inhibiting cytoplasmic and nuclear IKK, leading to NF-kβ inhibition [p<0.05].

After 8 weeks: We noticed that NF-kβ was more expressed in perisinusoidal area when compared to DMSO group. This expression returned more or less to control reactivity [Figure 5.d].

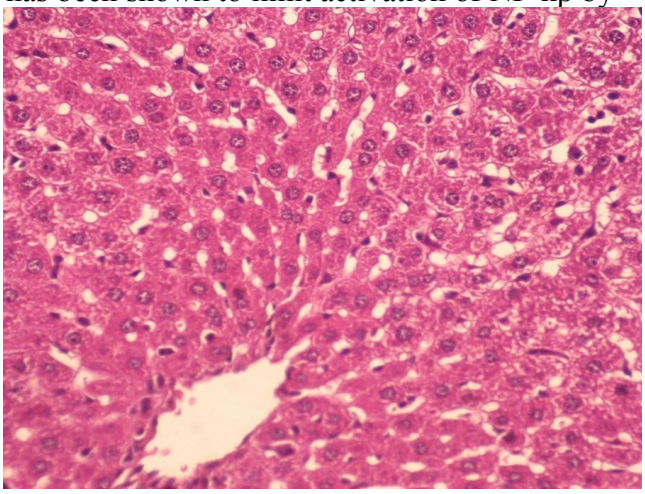

Figure 1.a

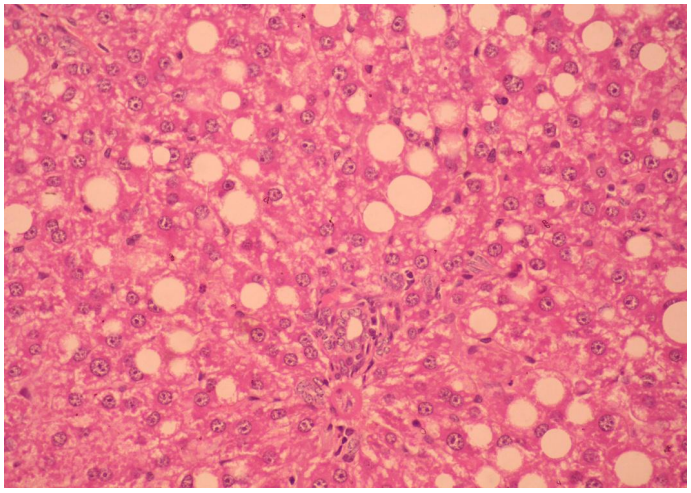

Figure 1.b

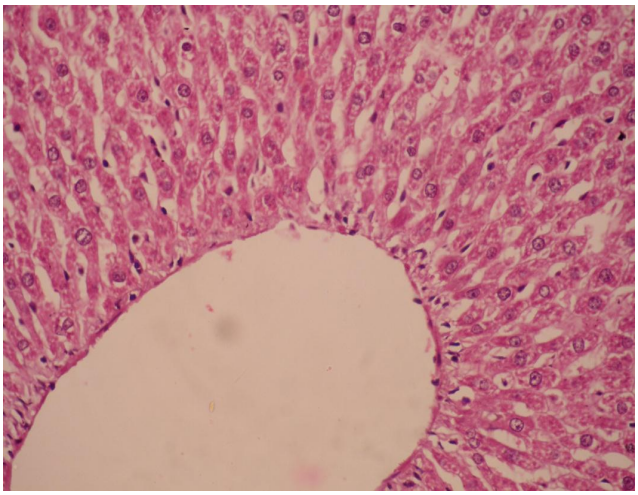

Figure 1.c

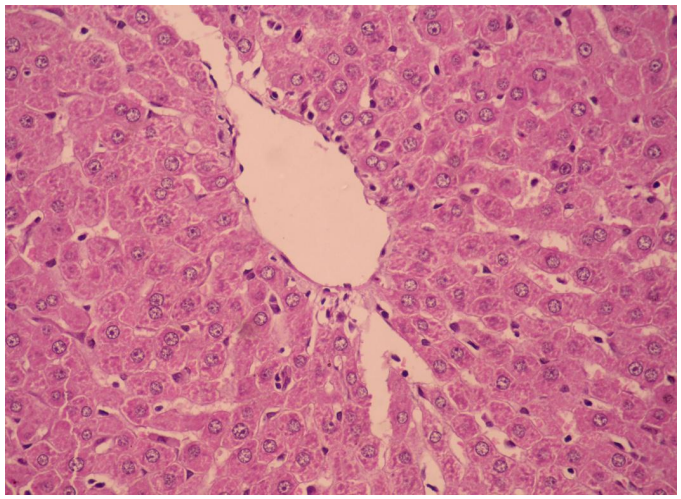

Figure 1.d

Figure 1: Liver tissue sections stained with Haematoxylin& Eosin [H&E] stain

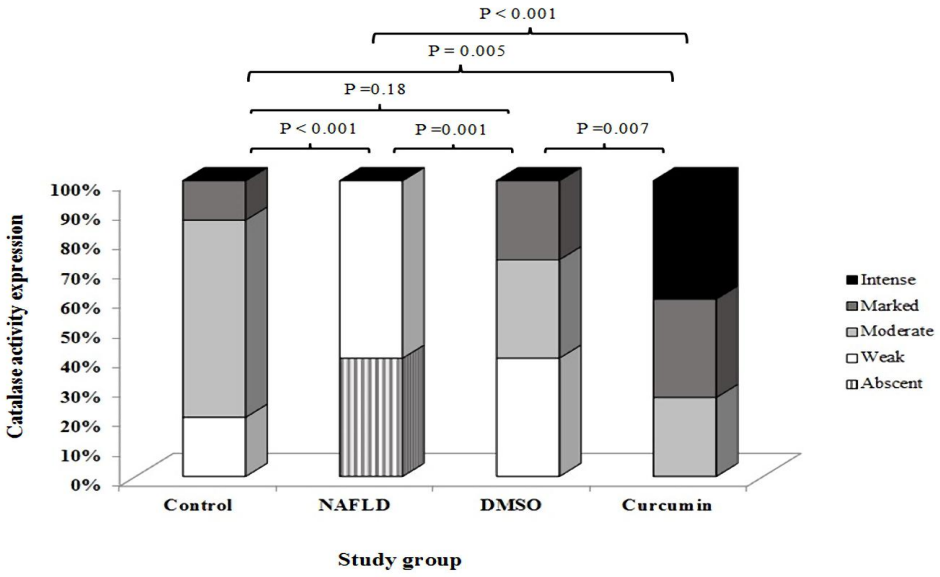

Figure 2: Arbitary stainability grades of catalase enzyme activity in liver tissues of gp I (control gp),gp II (NAFL gp), gp III (DMSO gp) and gp IV (curcumin gp)

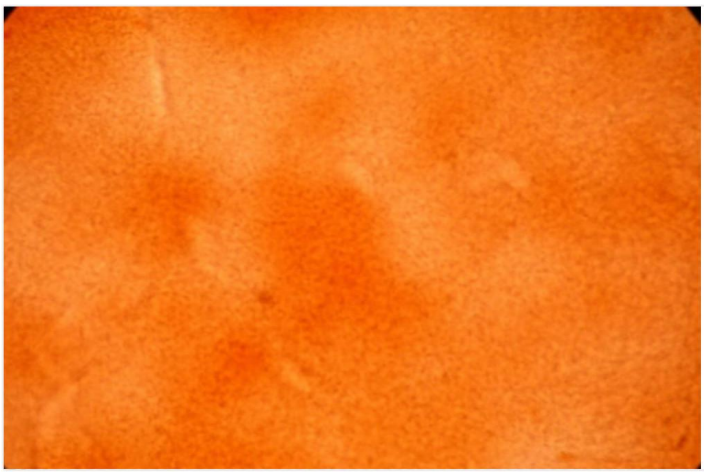

Figure 3.a

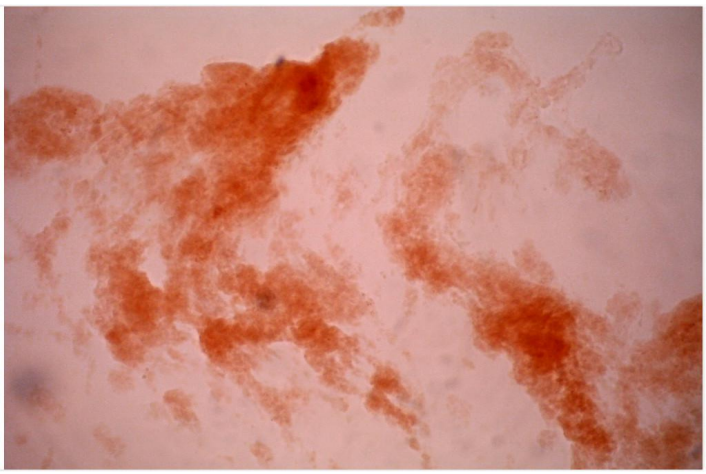

Figure 3.b

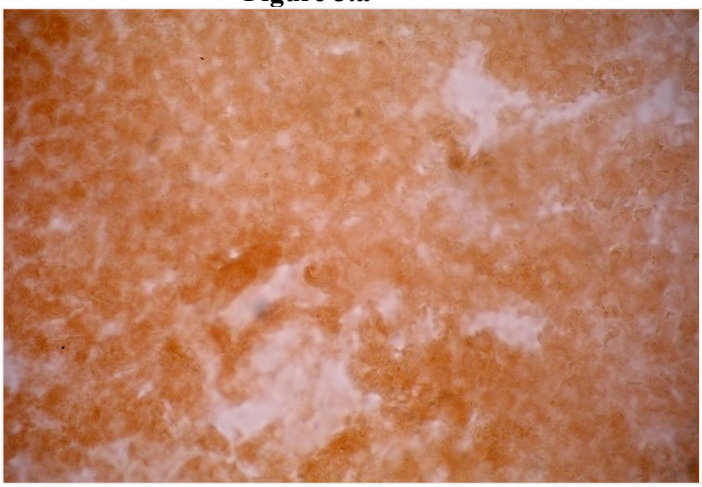

Figure 3.c

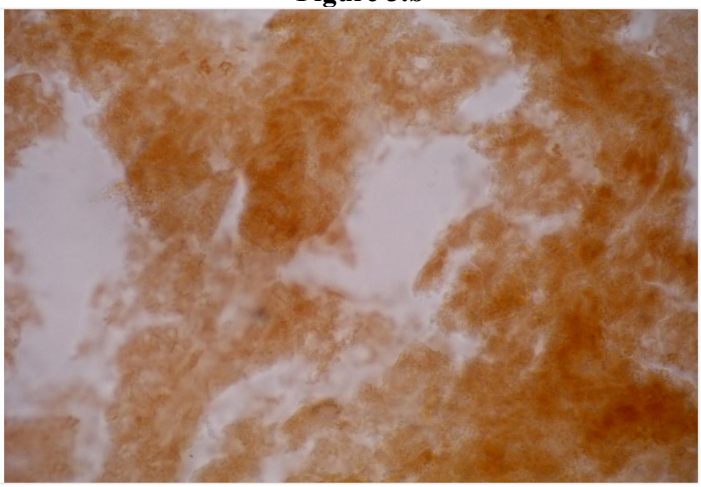

Figure 3.d

Figure 3: Catalase [Diaminobenzidine(DAB) method

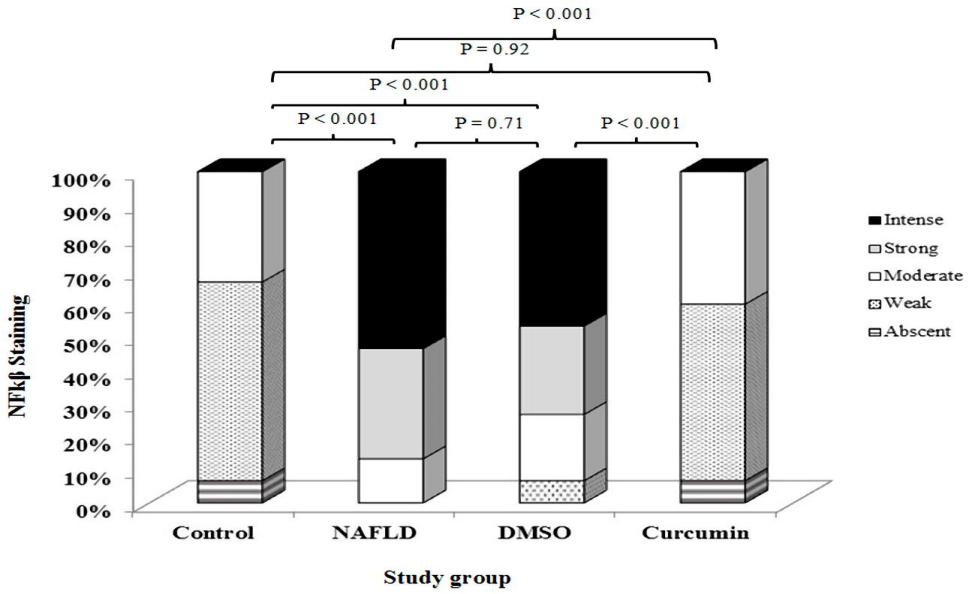

Figure 4 : Immunohistochemical staining of nuclear factor kappa B [NF-kB] expression scores in the 4 groups with their statistical comparisons

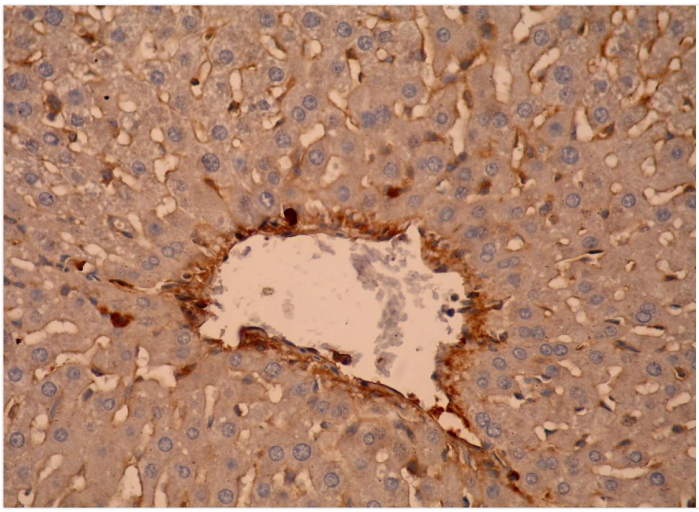

Figure 5.a

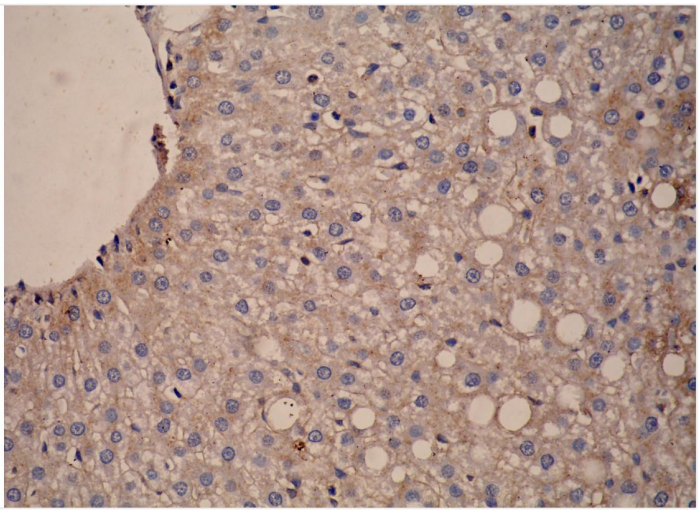

Figure 5.b

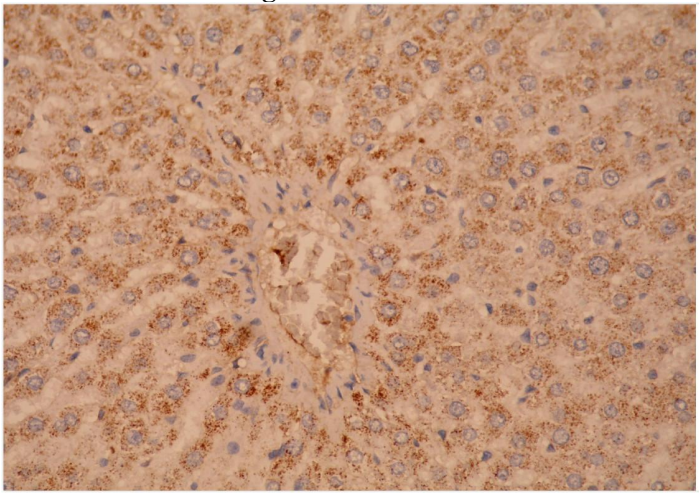

Figure 5.c

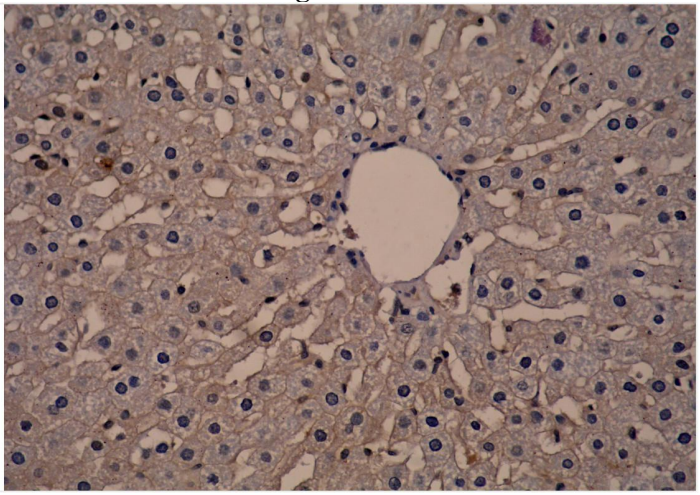

Figure 5.d

Figure 5: Immunohistochemical Results

5. Discussion

An important part in the pathogenesis of NAFLD is layed by insulin resistance, oxidative stress and the inflammatory cascade [15]. Experimantal evidence indicate that activation of proinflanmmatory pathways occurs mainly through NF-k β and the c-Jun N-terminal kinase (JNK) pathways which cooperate in inducing insulin resistance which, in association with several factors, including chronic inflammation, atherogenic dyslipidemia, hypercoagulation and hypofibrosis, hence, liver involvement occurs either as target of the metabolic abnormalities or as producer of proatherogenic molecules [16]. The present study demonstrates the potential value of curcumin in high-fat diet (HFD) induced NAFLD in rat models.

In addition, a study by Kempaiah and Srinivasan , reported that curcumin reduced membrane and intracellular lipid peroxide levels and induced antioxidant activity in hepatocytes and erythrocytes of rats on HFD [17] . Curcumin chelates and scavenges ROS and induces anti-oxidant enzymes [18, 19]. This decreases the oxidative stress for activation of NF-k β .

In our study, lipid content in liver was increased in NAFLD group compared to control group and decreased after curcumin treatment. The finding that curcumin reduces both hepatic and non-hepatic fat suggests that it lowers the fatty acid synthesis: oxidation ratio. Curcumin activates a key fatty acid oxidizing enzyme, acyl-CoA oxidase [20], a deficiency of which leads to hepatic steatosis.

This might be one-way curcumin prevents lipid accumulation. Tang and Chen reported that curcumin inhibits lipid accumulation in hepatic cells via AMPK activation[21]. In our study, histological examination of NAFLD rats liver samples revealed evidence of marked hepatic degeneration with the presence of micro and macrovesicular steatosis. We also noticed the congested portal tract and vacuolated cytoplasm in NAFLD rats compared to control rats which exhibited normal hepatic architecture and curcumin injection leads to histological picture apparently similar to the control group with hepatocyte regeneration. These results agrees well with previous characterization of the effects of HFD in inducing NAFLD [22].

The desired changes for experimental models of NASH includes steatosis, intralobular inflammation, ballooning of hepatocytes, and peri-sinusoidal fibrosis [23]. Samuhasaneeto et al [24], suggested that curcumin treatment improved liver histopathology by reduction of oxidation and inhibition of NF-k β activation in ethanol-related liver injury[24]. Curcumin ameliorates biochemical and histological indices of hepatic steatosis in many models of metabolic and dietary-induced steatosis and steatohepatitis. It attenuated the increase in hepatic and plasma total and VLDL triacylglycerols in normal rats fed a moderately high-fat [15 %] diet [20].

Our histochemical results of catalase in NAFL group revealed weak catalase activity compared to control group which showed dark brown granules of catalase distributed in the cytoplasm referring to increased activity. Curcumin treatment showed marked catalase activity compared with DMSO group at the same period. This finding is in agreement with a previous study showing that the curcumin treated group revealed a significant high catalase activity and that curcumin attenuates high fat diet induced kidney cortex derangement [25]. The antioxidant effect of curcumin was also confirmed in a study of Antioxidantand Anti-inflammatory Effects of Curcumin on CCl4-induced Liver Fibrosis in Rats [26].

Curcumin boosts the activity of several hepatic antioxidant enzymes, including catalase, superoxide dismutase and the glutathione system, both under normal and pathological conditions [27]. According to a previous study, catalase activity was decreased in NAFLD patients [28]. The abundant activation of NF-k β has been observed in obese patients and methionine choline-deficient diet-induced rats with NAFLD [29].

Earlier studies identified NF-k β as a key regulator of hepatic inflammatory recruitment and liver injury in NASH [30]. Curcumin has attracted attention for its prospective to inhibit NF-k β [31]. It suppresses NF-k β activation via direct modifications on the NF-k β /IkB complex and inhibition of IkB degradation that favors the retention of NF-k β into its inactive cytoplasmic form and interfere with NF-k β /DNA binding [19, 32].

The stimulation of hepatic NF-k β signaling is sufficient to increase the production of pro-inflammatory cytokines [33].The role of curcumin in obesity-associated inflammation [34] and steatohepatitis [4] has been established. In our study, the cells in NAFLD group showed significantly more expression for NF-k β P65 than those of the control group in the same period.

We also noticed weak reactivity in pericentral area of rat liver section and periportal area. Also, curcumin treatment inhibited the elevation of NF-k β P65, the number of NF-k β P65 positive cells in the curcumin group was significantly lower than that of the NAFLD and DMSO groups. With prolonged modeling time, the NF-k β P65 positivity of the cells returned more or less to control reactivity and was more expressed in perisinusoidal area in the curcumin group as compared with DMSO group. The present results are in line with a previous study exhibiting that curcumin prevented the degradation of inhibitor of NF-kB (IkB) in high fat diet induced kidney cortex derangement [25].

The inhibition of NF-k β signaling confers protection from obesity-induced inflammation in mouse models [35]. Curcumin has shown the ability to prevent hypertriglyceridemia and hepatic NF-k β activation [31]

Gukovsky et al [36], studied the pancreas in experimentally-induced pancreatitis of rats, reported that curcumin decreased inflammation by decreasing the activation of NF-k β [36].

Studies performed by Jacob et al [37], suggested that the anti-inflammatory effect of curcumin is mediated by the increasing PPAR- γ activation [37].

From the findings of our study, it can be concluded that curcumin can increase antioxidant defense mechanism by increasing activity of catalase enzyme and reduce inflammation through reducing the expression of NF-k β in the liver.

Conflict of Interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

This work has been presented as a Poster Presentation at The 15th ISOP Annual Meeting "Cubism in Pharmacovigilance" that was held In Prague, Czeck Republic 27-30 October, 2015.

This study had not been published but The Abstract ONLY was published as shown in the following.

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