Academic Sciences Asian Journal of Pharmaceutical and Clinical Research

Vol 5, Issue 4, 2012 ISSN - 0974-2441

Research Article

ROLE OF PIOGLITAZONE, QUERCETIN AND HYDROXY CITRIC ACID AGAINST NON ALCOHOLIC STEATOHEPATITIS (NASH) - HISTOLOGICAL AND SCANNING ELECTRON MICROSCOPY (SEM) STUDIES IN AN EXPERIMENTAL MODEL OF NASH

SURAPANENI KRISHNA MOHAN*[1], SARASWATHI. P[2] AND MALLIKA JAINU[3]

***[1]Assistant Professor, Department of Biochemistry, Saveetha Medical College & Hospital, Faculty of Medicine, Saveetha University, Saveetha Nagar, Thandalam, Chennai – 602 105, Tamilnadu, India.[2]Professor & Head, Department of Anatomy, Saveetha Medical College & Hospital, Faculty of Medicine, Saveetha University, Saveetha Nagar, Thandalam, Chennai – 602 105, Tamilnadu, India,[3]Assistant Professor, Department of Biomedical Engineering, SSN Engineering College, OMR, Klavakkam, Chennai – 603 110, Tamilnadu, India.**

Email: krishnamohan_surapaneni@yahoo.com

Received: 21 September 2012, Revised and Accepted:6 October 2012

ABSTRACT

Background: Non alcoholic steatohepatitis (NASH) one of the severe forms of the diseases that belongs to the spectrum of the Non alcoholic fatty liver disease (NAFLD). It is a silent asymptomatic disease which progress towards the fibrosis and cirrhosis, an end stage liver disease. Objective: To study the protective role of pioglitazone, quercetin and hydroxy citric acid against NASH by virtue of the histopathological studies and scanning electron microscopic studies. Materials & Methods: A portion of the liver tissue from male wistar rats was taken and used for histopathological and scanning electron microscopic studies. Results: The results of our study confirmed the induction of NASH in rats fed with high fat diet for 8 weeks evidenced by the diffused fatty infiltration of hepatocytes with mono nuclear inflammatory infiltrate. Experimental NASH treated with pioglitazone showed no fatty degeneration, with quercetin was observed normal hepatocytes no obvious fatty & inflammatory changes are seen and with hydroxy citric acid there was local hepatocyte necrosis with inflammatory collections. Rats fed with standard diet simultaneously with pioglitazone (pioglitazone control), quercetin (quercetin control), and hydroxy citric acid showed normal hepatocytes and no pathological changes were observed. These histopathological studies were supported by the results of the scanning electron microscopic studies. Conclusion: The histopathological evidence of experimental NASH in animals indicated that progressive disease was associated with the presence of diffused fatty infiltration of hepatocytes with mono nuclear inflammatory infiltrate whereas, quercetin treated groups showed normal hepatocytes and no obvious fatty & inflammatory changes are seen. The severity of experimental NASH was reduced after treatment with pioglitazone and hydroxy citric acid. The results of the histopathological studies were supported by the scanning electron microscopy (SEM) studies.

Keywords: NASH, high fat diet, quercetin, hydroxy citric acid, pioglitazone, SEM.

INTRODUCTION

Non alcoholic steatohepatitis (NASH), one of the severe forms of the diseases that belongs to the spectrum of the Non alcoholic fatty liver disease (NAFLD) [1]. Non alcoholic steatohepatitis is an asymptomatic disease, but is severe which may lead to the cirrhosis if uncured [2]. Cirrhosis is an end stage liver disease which ultimately leads to death of the individual [3]. The incidence of NASH is increasing year by year worldwide and is forming one of the major causes of deaths due to the liver failure [4]. Liver biopsy is the only way to confirm the presence or absence of NASH and is an invasive technique [5], but is considered as the gold standard. Since NASH is an asymptomatic disease and since liver biopsy technique is an invasive technique, many patients will not accept to go for the liver biopsy, to confirm the presence or absence of NASH [6]. This hinders the researchers to continue the studies in NASH to invent non-invasive markers for the diagnosis of NASH and to design various treatment strategies for NASH. Hence, as part of our phase I studies, we have successfully established a model of NASH induced in Rats [7]. In our phase II studies, we have selected three drugs to compare the role of these three drugs viz. pioglitazone, quercetin and hydroxy citric acid in experimentally induced rats.

MATERIALS AND METHODS

Male Wistar rats weighing approximately 250g were housed in solid-bottomed polypropylene cages under strict veterinary supervision and maintained in control rooms with 12 h light/dark cycle. The animals received commercial rat diet, standard diet, high-fat diet and water *ad libitum* as per the experimental protocol. This study conformed to the guiding principles of Institutional Animal Ethical Committee (IAEC), Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Guide for the care and use of laboratory animals (IAEC Approval Number: 001/006/2010 & 01/007/2011).

Experimental drug treatment protocol for phase – II study, in the experimental model of nash:

The Male wistar rats selected for the study were divided into 8 groups as follows:

Group 1; Controls (n = 6): Control rats received regular standard diet for 8 weeks.

Group 2; NASH (n=6): Rats induced with NASH by feeding high fat diet for 8 weeks.

Group 3; Pioglitazone Control (n=6): Rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of Pioglitazone (4 mg/kg. b.wt) (0.5% methyl cellulose w/v) for further 4 weeks.

Group 4; Quercetin Control (n=6): Rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of quercetin (20 mg/kg. b.wt) dissolved in 1% DMSO v/v for further 4 weeks.

Group 5; Hydroxy Citric Acid Control (n=6): Rats fed with standard diet for 4 weeks, then feeding them with standard diet simultaneously with intra gastric administration of hydroxy citric acid (150 mg/kg. b.wt) for another 4 weeks.

Group 6; NASH + Pioglitazone (n=6): Rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of Pioglitazone (4 mg/kg. b.wt) (0.5%methyl cellulose w/v) for another 4 weeks.

Group 7; NASH + Quercetin (n=6): Rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intragastric administration of quercetin (20 mg/kg. b.wt) dissolved in 1% DMSO v/v for another 4 weeks.

Group 8; NASH + Hydroxy Citric Acid (n=6): Rats fed with high fat diet for 4 weeks, then feeding them high fat diet simultaneously with intra gastric administration of Hydroxy Citric Acid (150 mg/kg. b.wt) for another 4 weeks.

After the experimental period, the animals were sacrificed after 12 h of fasting by cervical decapitation and blood was collected, centrifuged for 5 min at 3000 rpm/min, and serum was stored at −70 °C from that various biochemical analysis were carried out.

Liver tissues of all the experimental groups were dissected out and fixed in 10% buffered neutral formalin solution for histological studies. The liver tissues were dissected out immediately and washedin ice-cold saline. Hundred milligrams of liver tissue was weighed accurately and homogenized in 5ml of 0.1MTris–HCl buffer (pH 7.4) in ice-cold condition. The homogenate was centrifuged at 2500×g and the clear supernatant solution was taken for the analytical procedures.

Histological Studies

Liver tissue samples from each group were fixed in phosphate buffered formalin for 24 h. The formalin fixed specimens were embedded in paraffin, sectioned (3-5µm) using microtome and stained with haematoxylin and eosin dye. The histological sections were evaluated by light microscopy at 20X magnification.

Scanning Electron Microscopic Studies (SEM)

Animals were sacrificed after light anesthesia with sodium pentobarbital. The thin slices of liver tissues were dissected under an operating microscope, cut longitudinally and placed in 2.5% glutaraldehyde in tyrodes solution for atleast 5 days. The liver tissues were stiff and maintained their shape and dimension on dissection.

The liver samples were stained with osmium, tetroxide dehydrated with graded ethanol series and propylene oxide and ortical point dried. The mounted and sputter coated with gold Model 5150 B sputter coater (Edwards High Vacuum Crawley U.K). Samples were examined under a (Philips SEM 505 scanning electron microscope (Phillip, Eindhoven, Netherlands). The entire endothelial surface was observed at low magnification and all areas of abnormal endothelium and lesions recorded at magnification upto 3800 X.

RESULTS

The results of the histopathological studies conducted to create experimental model of NASH in rats have been shown in Fig. 1 and 2.

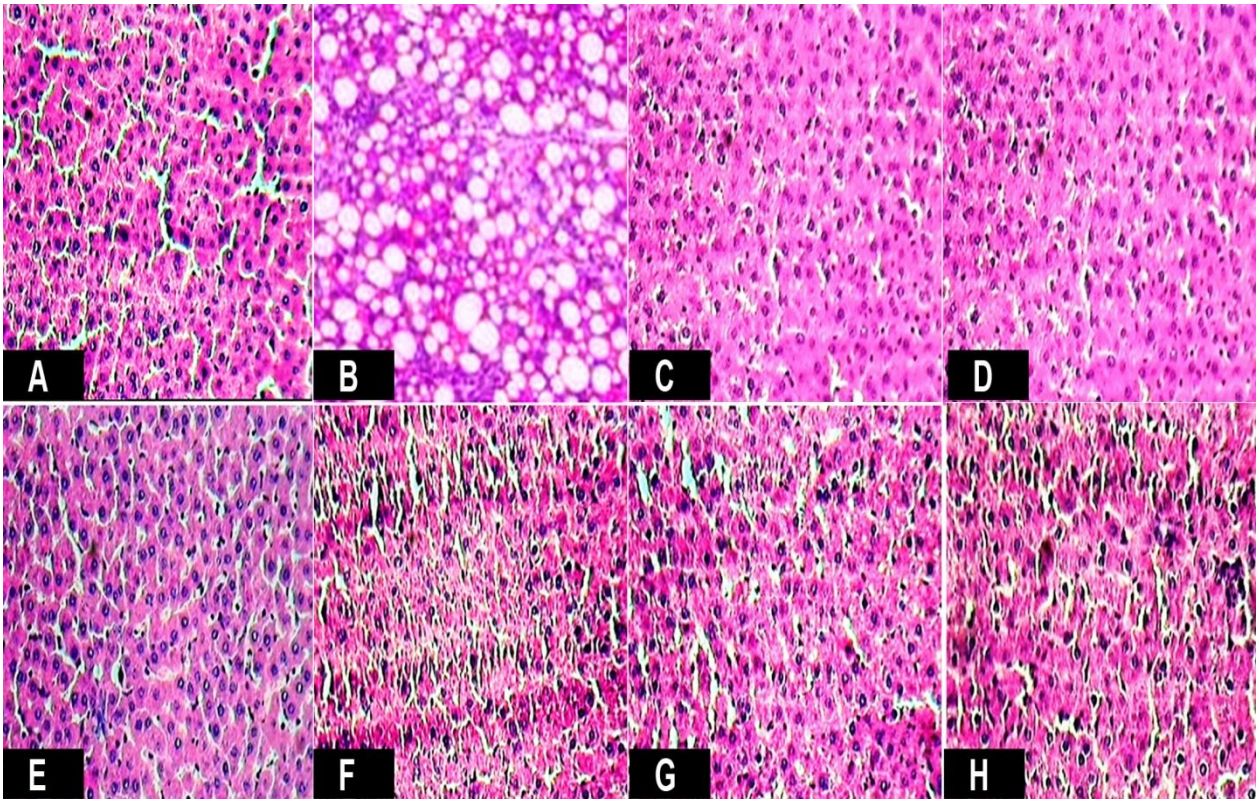

Fig. 1 (A-H): histopathological studies (comparative study of role of pioglitazone, quercetin and hydroxy citric acid experimental model of NASH).

Fig. 1A: Group 1 Control - Showed NORMAL Hepatocytes;
Fig. 1B: Group 2 NASH (High Fat Diet) - Showed Diffused Fatty Infiltration of Hepatocytes with Mono Nuclear Inflammatory Infiltrate;
Fig. 1C: Group 3 Pioglitazone Control - Shows NORMAL Hepatocytes; no pathological changes observed;
Fig. 1D: Group 4; Quercetin Control - No Pathological Changes, Shows NORMAL Hepatocytes;
Fig. 1E: Group 5; Hydroxy Citric Acid Control - No Pathological Changes, Shows NORMAL Hepatocytes;
Fig. 1F: Group 6; NASH + Pioglitazone - No Fatty Degeneration is seen;
Fig. 1G: Group 7; NASH + Quercetin- Hepatocytes appear Normal. No Obvious Fatty & Inflammatory Changes are seen;
Fig. 1H: Group 8; NASH + Hydroxy CitricAcid- Local Hepatocyte Necrosis is observed

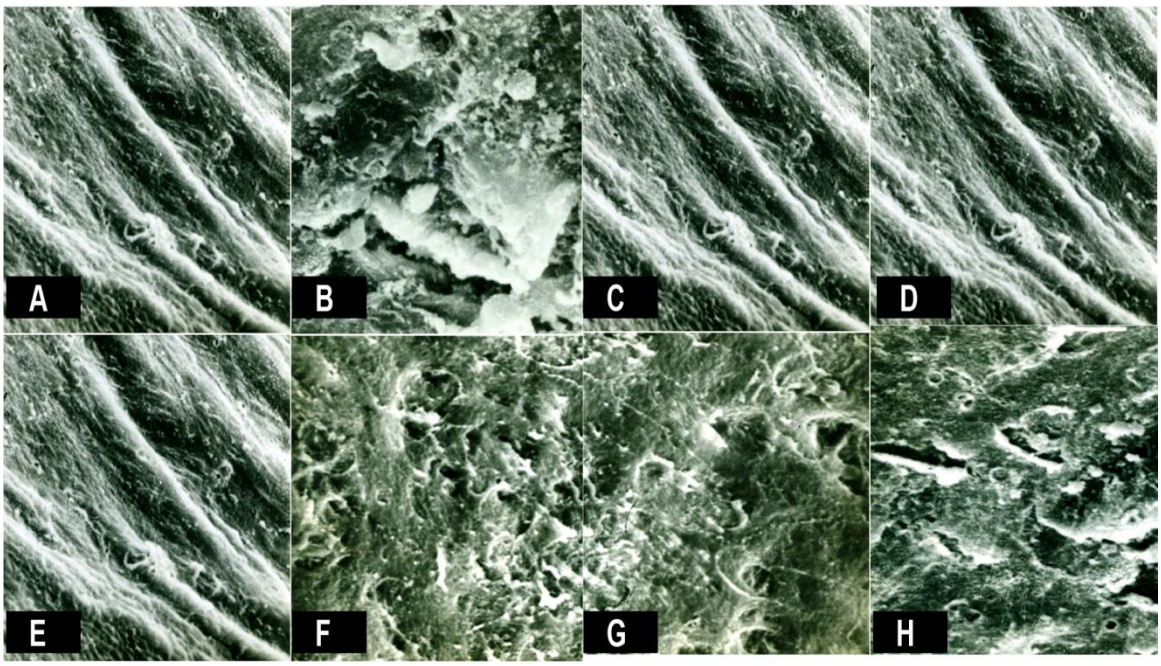

Fig. 2 (a-h): Scanning Electron Microscopy (SEM) studies (comparative study of role of pioglitazone, quercetin and hydroxy citric acid experimental model of NASH).

Fig. 2A: Group 1 Control - Liver section of rat showing epithelium and normal layers with normal architecture;
Fig. 2B: Group 2 NASH (High Fat Diet) - Liver section of NASH rat showing accumulation of fat on liver tissues, with cellular destruction showing pathological changes;
Fig. 2C: Group 3 (Pioglitazone Control) - Liver section of rat showing epithelium and normal layers with normal architecture;
Fig. 2D: Group 4 (Quercetin Control) - No pathological changes observed and showed normal layers with normal architecture;
Fig. 2E: Group 5 (Hydroxy Citric Acid Control) - No pathological changes observed and showed normal layers with normal architecture;
Fig. 2F: Group 6 (NASH + Pioglitazone) - Liver section of NASH rat showing marked reduction in the size of fat accumulation;
Fig. 2G: Group 7 (NASH + Quercetin) - Hepatocytes appear Normal & No Obvious Fatty & Inflammatory Changes are seen;
Fig. 2H: Group 8 (NASH + Hydroxy CitricAcid) - Local Hepatocyte Necrosis with Inflammatory collections is seen but showed reduction in the size of fat accumulation

DISCUSSION

Currently, no specific therapies for NASH exist, there is a link between NASH and insulin resistance, two types of drug, which are sometimes called 'insulin sensitisers', have been tried. These are biguanides (metformin) and the glitazones (rosiglitazone and pioglitazone). Thiazolidinediones, such as pioglitazone, is an oral antidiabetic agent that acts primarily by decreasing insulin resistance, are synthetic ligands for peroxisome proliferator-activated receptors (PPARs). Pioglitazone (PIO) hydrochloride is a widely used drug in the treatment of insulin resistance diabetes [8].

For the past several years, small and complex molecules have been isolated from the various species of *Garcinia,* which include xanthones and xanthone derivatives [9]. However, the isolation of (-)-hydroxycitric acid [(-)- HCA] from a few species of *Garcinia* and its biological properties have attracted the attention of biochemists and health practitioners [10]. The physiological and biochemical effects of (-)-HCA have been studied extensively for its unique regulatory effect on fatty acid synthesis, lipogenesis, appetite, and weight loss [11]. Overall, antioxidants represent a novel class of medications that have shown some promising initial results in the treatment of NASH [11].

Quercetin is a plant-derived substance, or phytochemical, known as a flavonoid. Emerging research suggests quercetin may reduce the risk of upper respiratory tract infection during intense physical exercise, which is likely attributable to its antioxidant, anti-inflammatory and anti-pathogenic effects [12]. Quercetin offers a variety of potential therapeutic uses primarily in the prevention and the treatment of the conditions [12].

Since the pathogenesis of NASH involved interplay of 3 possible mechanisms such as hyper insulinemia, lipotoxicity and oxidative stress [13], we have chosen 3 categories of drugs, pioglitazone as insulin sensitizer, quercetin as hepatoprotectant & antioxidant and hydroxy citric acid as a lipid lowering & antiobesity agent. The experimental model of NASH in rats by feeding high fat diet for 8 weeks was used to conduct a comparative study of role of pioglitazone, quercetin and hydroxy citric acid on various parameters.

Histological evaluation remains the sole method of distinguishing steatosis from advanced forms of NAFLD, i.e. nonalcoholic steatohepatitis (NASH) and fibrosis. Histologic examination of livers from high fat diet demonstrated substantial steatosis with inflammatory changes, micro vesicular and macrovesicular steatosis were clearly visible. The macrophages are massively recruited into the liver upon toxic injury and may differentiate into fibrocytes [14, 15]. NASH histopathology has been reported to have panacinar or periportal steatosis, rare ballooning and portal tract expansion by chronic inflammation or fibrosis [16]. Neutrophilic cells in lobular inflammatory infiltrate are a distinguishing feature of steatohepatitis. Histologically, fat deposition is typically macrovesicular and inflammation of steatohepatitis is predominantly lobular. Neutrophilic cells in lobular inflammatory infiltrate are a distinguishing feature of steatohepatitis and differentiate it from other chronic hepatitis [17]. Steatohepatitis, which is characterized by steatosis, inflammation, and cell injury, i.e. NASH. Inflammation consists of a mixed inflammatory cell infiltrate, composed of lymphocytes, some eosinophils and, occasionally, a few neutrophils [16, 18].

Histological studies showed distinguishing disease progression and regression of NASH was measured after the treatment of pioglitazone, quercetin and hydroxyl citric acid. Our present investigation showed that, in experimental NASH the progressive disease was associated with the presence of diffused fatty infiltration of hepatocytes with mono nuclear inflammatory

infiltrate, quercetin treated groups showed normal hepatocytes and no obvious fatty & inflammatory changes are seen which may be due to the anti-inflammatory effect of quercetin [19]. The severity of experimental NASH was reduced after treatment with pioglitazone and hydroxy citric acid was also observed. Pioglitazone therapy was associated with a reduction in these histologic predictors of progressive disease. Therefore, long-term pioglitazone therapy may arrest or reverse the progression of NASH and improve its clinical outcome. Treatment with pioglitazone was associated with a reduction in steatosis, injury lobular inflammation [20].

SEM studies revealed accumulation of fat on liver tissues, with cellular destruction showing pathological changes in experimental NASH group, whereas in pioglitazone & hydroxy citric acid treated against NASH showed reduced deposition of fat in liver tissues. Quercetin showed protective role with no pathological inflammatory changes in liver tissues which may be due to hepatoprotective & antioxidant activity. The results of the histopathological studies were supported by the scanning electron microscopy (SEM) studies.

CONCLUSION

The severity of NASH was reduced by co-treatment with pioglitazone, hydroxy citric acid and quercetin which are clearly evident in histological & SEM studies. Quercetin showed more protection when it is compared with pioglitazone & hydroxy citric acid against NASH, so this drug can be considered for further advanced studies to understand the mechanism of its protective action.

REFERENCES

1. Adams LA, Lymp JF, Sauver JS, et al. The natural history of nonalcoholic fatty liver. Gastroenterology 2005;129:113–121.
2. Harrison SA, Neuschwander-Tetri BA. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Clin Liver Dis 2004; 8:861–879.
3. Takashi Matsuzaka and Hitoshi Shimano. Molecular mechanisms involved in hepatic steatosis and insulin resistance. *J Diabetes Invest,* 2011; 2(3): 170–175.
4. Beymer C, Kowdley KV, Larson AA, et al. Prevalence and predictors of asymptomatic liver disease in patients undergoing gastric bypass surgery. Arch Surg 2003;138:1240 –1244.
5. Ratziu V, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, Grimaldi A, Capron F, Poynard T. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology, 2005; 128: 1898-1906.
6. Harrison SA, Neuschwander-Tetri BA. Nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Clin Liver Dis 2004; 8:861–879.
7. Surapaneni Krishna Mohan., Saraswathi P and Mallika Jainu. Non alcoholic steatohepatitis (NASH) experimental model induction in rats. Int J Pharm Bio Sci, 2012 July; 3(3): (B) 1085 – 1090.
8. Jagdish kakadiya, Nehal shah, effect of some synthetic and herbel drugs on tumer necrosis factor alpha in renal reperfusion induced renal damage in type 2 diabetic rats. International journal of preclinical and pharmaceutical Research, 2011; 2(1): 30-37.
9. Bennet, GJ, Lee HH. Xanthones from Guttiferae. Phytochemistry, 1989; 28: 967-998.
10. Rama Rao AV, Venkataswamy G, Yemul SS. Xanthochymol & isoxanthochymol; two polyisoprenylated benzophenoes from Garcinia xanthochymus. Ind. J. Chem, 1980; 19: 627- 633.
11. Minami H, Kinoshita M, Fukuyama Y, Kodama M, Yoshizawa T, Sugiura M, Nakagawa K, Tago H. Antioxidant xanthones from *Garcinia subliptica*. *Phytochemistry,* 1994; 36; 501-506.
12. Alexandra B. Bentz. A Review of Quercetin: Chemistry, Antioxidant Properties, and Bioavailability, Journal of Young Investigators, 2009; 19 (10): retrieved 6 October 2012 from http://www.jyi.org/research/re.php?id=3416.
13. Cusi K. Non-alcoholic fatty liver disease in type 2 diabetes mellitus. Curr Opin Endocrinol Diabetes Obes 2009;16:141–9.
14. Musso G, Gambino R, De Michieli F, Cassader M, Rizzetto M, Durazzo M, et al. Dietary habits and their relations to insulin resistance and postprandial lipemia in nonalcoholic steatohepatitis. HEPATOLOGY 2003;37:909-916.
15. Niedermeier M, Reich B, Rodriguez Gomez M, Denzel A, Schmidbauer K, Gobel N, et al. CD4þ T cells control the differentiation of Gr1þ monocytes into fibrocytes. Proc Natl Acad Sci USA 2009;106:17892-17897.
16. Brunt EM. Histopathology of non-alcoholic fatty liver disease. Clin Liver Dis 2009; 13:533-544.
17. Das K and Kar P. Update Article: Non-Alcoholic Steatohepatitis, JAPI, 2005; 53: 195-199.
18. Hübscher SG. Histological assessment of non-alcoholic fatty liver disease. Histopathology. 2006;49:450–465.
19. Mari M, Fernandez-Checa JC. Sphingolipid signalling and liver diseases. Liver Int, 2007; 27: 440–450.
20. Aithal GP, Thomas JA, Kaye PV, Lawson A, Ryder SR, et al, Randomized, Placebo-Controlled Trial of Pioglitazone in Nondiabetic Subjects With Nonalcoholic steatohepatitis,Gastroenterology, 2008; 135: 1176–1184.
21. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*. 2005; 41:1313–1321.
22. Powell EE, Cooksley WG, Hanson R, et al. The natural history of nonalcoholic steatohepatitis : A follow-up study of forty two patients for up to 21 years. Hepatology 1990;11:74.
23. Rohit Kohli, Michelle Kirby, Stavra A. Xanthakos, Samir Softic, Ariel E. Feldstein, Vijay Saxena, Peter H. Tang, Lili Miles, Michael V. Miles,William F. Balistreri, Stephen C. Woods, and Randy J. Seeley, High-Fructose, Medium Chain Trans Fat Diet Induces Liver Fibrosis and Elevates Plasma Coenzyme Q9 in a Novel Murine Model of Obesity and Nonalcoholic Steatohepatitis. *Hepatology,* 2010; 52: 934-944.