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Voluntary oral feeding of rats not requiring a very high fat diet is a clinically relevant animal model of non-alcoholic fatty liver disease (NAFLD)

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Summary. Animal models used to study the pathogenesis of non-alcoholic fatty liver disease (NAFLD) are, in general, either genetically altered, or fed with a diet that is extremely high in fat or carbohydrates. Recent findings support the role of oxidative stress, lipid peroxidation and inflammation as probable causative factors. We hypothesize that not only the amount of dietary fat, but the quality of fat is also important in inducing NAFLD. Based on previous observations that female rats fed a diet comprising unsaturated fatty acids are susceptible to liver injury, we proposed that female rats fed with a diet containing fish oil and dextrose would develop pathological and biochemical features of NAFLD. We fed a highly unsaturated fat diet (30% fish oil) to female Sprague-Dawley rats (180-200g), consumed ad libitum for 8 weeks (NAFLD; n=6-8). Control animals (CF; n=6-8) were fed with an isocaloric regular rat chow. At killing, blood and liver samples were collected for serum alanine aminotransferase (ALT), histology and molecular analysis. Each histological sample was evaluated for fatty liver (graded from 0 to 4+ according to the amount of fatty change), necrosis (number of necrotic foci (no./mm^2) and inflammation (cells per mm^2). The amount of collagen formation was estimated based on the amount of Sirius Red staining. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out for tumor necrosis factor alpha (TNF-α), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), adiponectin, glutathione peroxidase (GPx), superoxide dismutase (Cu/Zn SOD) and catalase (CAT). Western Blot analysis was done for cyclooxygenases-2 (COX-2), inducible nitric oxide synthase (iNOS) and nitrotyrosine. Electrophoretic mobility shift assay was performed for nuclear factor-kappa B (NF-κB) activity. NAFLD rats had a significantly higher serum ALT level, amount of collagen formation, fatty liver, necrosis and inflammation when compared with the chow-fed control rats. mRNA and protein levels of NF-κB regulated genes, which included TNF-alpha, COX-2 and iNOS were also significantly ($p<0.01$; $p<0.01$; $p<0.05$ respectively) upregulated in the NAFLD group when compared with the chow-fed control rats. mRNA levels of antioxidants CAT and GPX were reduced by 35% and 50% respectively in the NAFLD group. However, Cu/Zn SOD mRNA was similar in both groups. The mRNA level of adiponectin was also reduced in NAFLD group. NF-κB activity was markedly increased in the NAFLD rats ($p<0.01$). The level of oxidative stress, represented by the formation of nitrotyrosine, was significantly elevated in the NAFLD rats ($p<0.01$). We conclude that NAFLD rats demonstrated several features of NAFLD, which included fatty liver, inflammation, necrosis, increased oxidative stress, an imbalance between pro and antioxidant enzymes mRNAs, reduced adiponectin levels and upregulation of pro-inflammatory mediators. We propose that female rats fed with a diet containing highly unsaturated fatty acids are an extremely useful model for the study of NAFLD.

Key words: Tumor necrosis factor, Cyclooxygenase, Glutathione peroxidase, Superoxide dismutase, Catalase

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Introduction

Current animal models for the study of non-alcoholic fatty liver disease (NAFLD) are usually either genetically altered animals or fed with a diet that is extremely high in fat or carbohydrates. Generally, two feeding methods are employed in animal models; voluntary and intragastric. Intragastric rat feeding

models have contributed to a greater understanding of alcoholic liver disease (Nanji et al., 2001) and more recently in non-alcoholic fatty liver disease (NAFLD) (Zou et al., 2006). A voluntary route of consumption of diet instead is the preferred means of food intake because it requires no special training and additional technical equipment. Ad libitum consumption of diet, however, tends to be self-limiting, either because of the diet composition or palatability (Corbertt et al., 1986; Maegawa et al., 1986; Watarai et al., 1988; Lieber et al., 2004). This is contrary to what is observed in the clinical setting, where over indulgence in eating is the usual occurrence and therefore obesity is usually the end result. Some investigators, however, have found it difficult to induce obesity in normal rats using a high fat diet fed ad libitum (Maegawa et al., 1986; Pedersen et al., 1991). Nevertheless, some studies were successful in showing features of development of non-alcoholic fatty liver disease (NAFLD), employing a diet with a content of more than 70 percent fat. This fat is very different from what is described as a "westernized diet". (Lieber et al., 2004; Zou et al., 2006).

Non-alcoholic fatty liver disease (NAFLD) is one of the liver diseases that require precise clinicopathological correlation for diagnosis (Brunt, 2001). It has been considered a relatively benign disease in the past, but more recent studies suggest a more progressive and sinister course in the long term (Caldwell et al., 1999; Poonawala et al., 2000; Reid, 2001). Among the many causative factors of NAFLD, oxidative stress, lipid peroxidation and inflammation are considered the most probable causative factors. The prevailing concept is initially a "two hit" and more recently a "multiple hit" hypothesis (Day and James, 1998; McCullough, 2006). The first hit is largely the result of insulin resistance that leads to a reversible accumulation of fat in hepatocytes. (Chitturi et al., 2002). This accumulation of lipid within hepatocytes is due to (i) increased delivery and uptake into liver cells, as well as increased de novo synthesis of liver fatty acids and triglycerides, and (ii) failure of very low-density lipoprotein synthesis and triglyceride export, and impaired hepatic mitochondrial ß-oxidation (Anstee and Goldin, 2006). The second or subsequent hits involve a combination of oxidative stress, lipid peroxidation, cell death and pro-inflammatory cytokine mediated liver injury (Anstee and Goldin, 2006). Pivotal to this concept is the relative vulnerability of the hepatocytes to injury i.e. histological progression from steatosis to steatohepatitis.

In liver disease with similar pathologic features, alcoholic liver disease (ALD), it has been shown that not only the quantity but also the quality of fat is important (French et al., 1991). A more unsaturated diet such as fish oil when compared to corn oil, leads to higher levels of lipid peroxidation and the expression of chemokines and cytokines (Nanji, 2004a). Some investigators have successfully developed NAFLD models in rats by using a corn (unsaturated) oil (Lieber et al., 2004; Zou et al., 2006). We hypothesized that feeding diet containing levels of fat similar to those consumed in humans but containing a more unsaturated fat could achieve a similar effect to those animals fed with an extra high fat diet. These effects include pathologic changes seen in NAFLD and NASH, including enhanced binding activity of NF-κB, concentration of proinflammatory markers, and markers of oxidative stress. The aim of the present study is to produce a clinically relevant animal model of non-alcoholic fatty liver disease (NAFLD) not requiring a high fat diet through voluntary oral feeding.

Materials and methods

Animal experiment

The diet provided 30% of its energy from fat, 35% from carbohydrate and 35% from protein. Supplements of vitamins and minerals were included as described previously (Tipoe et al., 2008). Adult female Sprague-Dawley rats were handled and cared for in compliance with the requirements of The University of Hong Kong and the National Institute of Health guidelines. The Laboratory Animal Unit of The University of Hong Kong is fully accredited by the Association for Assessment and Accreditation for Laboratory Animal Care (AAALAC International).

The rats (n=-8), weighing 180-200g, were divided into two groups: NAFLD group and control (CF) group. The rats in NAFLD group were fed ad libitum with a diet high in unsaturated fat (30% fish oil) for eight weeks. The composition of the liquid diet included fish oil (Sigma-Aldrich Co., MO, USA), mineral mix (Dyets Inc., USA), vitamin mix (Dyets Inc., USA), choline bitartrate (Dyets Inc., USA), dextrose, food grade (Dyets Inc., USA), DL-methionine (Bio-Serv, USA) and lactalbumin hydrolysate (Bio-Serv, USA). The control group was fed with an isocaloric regular rat chow. Blood samples were obtained by cardiac puncture. A portion of liver samples was fixed in formalin and the remainder frozen at -70°C for further analysis.

Histopathological analysis and Sirius Red staining for collagen

Liver samples were obtained when the rats were killed and formalin fixed. Hematoxylin and eosin stain was used for light microscopy. The severity of liver pathology was assessed as follows: steatosis (the % of liver cells containing fat), 1+, ≤ 25% of cells; 2+, 26-50% of cells; 3+, 51-75% of cells; and 4+, >75% of cells. Necrosis was quantified as the number of necrotic foci per square millimeter, and inflammation was scored as the number of inflammatory cells per square millimeter. At least three different sections were examined per sample of liver. The pathologist evaluating these sections was unaware of the treatment the rats had received (Nanji et al., 2001)

For evaluation of fibrosis around the central veins and surrounding regions, sections were stained with Sirius Red. The area of collagen deposition around each central vein and surrounding regions was measured

using an image analyzer Leica QWIN (Cambridge, UK). The percent area of collagen deposition was calculated by dividing the sum areas of the Sirius Red stained over the sum areas of the reference fields multiplied by 100.

Determination of alanine aminotransferase (ALT) activity in serum

Alanine aminotransferase activity in serum was used as a biochemical indicator of hepatic injury. A reaction mixture containing L-alanine (80 mmol/L), NADH (0.2 mmol/L), and lactate dehydrogenase (2 units) in potassium phosphate buffer (0.2 mmol/L, ph7.4) and serum (0.35 mL) was incubated at 37°C for 3 min to determine the basal rate of NADH consumption at 340 nm. α-ketoglutarate (10 mmol/L) was subsequently added to measure the rate of NADH ultilization by alanine aminotransferase present in serum. The activity of the enzyme was expressed in units per liter (U/L) of serum.

RNA extraction and analysis of messenger RNA by reverse transcriptase-polymerase chain reaction

To determine the mRNA expression level of iNOS, COX-2, adiponectin, TNF-α, SOD, GPX and CAT genes in the liver, total RNA was extracted using RNeasy mini kits (Qiagen Inc, Valencia, CA, USA). Reverse transcription and amplification by polymerase chain reaction (PCR) were performed as described previously (Tipoe et al., 2006). The sequence of primer pairs, 5' and 3', and the predicted size of the amplified polymerase chain fragments have been previously determined (Tipoe et al., 2006; Hung et al., 2008). The primer sequences for the amplification of adiponectin were 5'-AATCCTGCCCAGTCATGAAG-3' and 5'-CATCTCC TGGGTCACCCTTA-3'. The corresponding PCR was carried out at an annealing temperature of 55°C for 38 cycles with a product size of 220bp. The PCR products were subjected to electrophoresis and ethidium bromide staining. The intensity of the PCR products was analyzed by densitometry using an image analyzer (Image J, National Institute of Health, Maryland, USA). GAPDH was used as internal control.

Western Blot analysis

The specificity and the relative differences of iNOS protein expression were confirmed by Western blot analysis as previously described (Tipoe et al., 2006). Briefly, liver tissue was rapidly homogenized and lysed in 5 volumes of cold RIPA lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) on ice for 30 minutes. The cell debris was removed by centrifuging at 1,300 rpm for 30 minutes at 4°C. The protein concentration in the supernatant was determined using BCA protein assay kit (Pierce, Rockford, IL, USA). Polyacrylamide gel electrophoresis using 20 μg of total protein was performed using the Mighty Small II run gel system (SE 250/260, Hoefer, Pharmacia Biotech Inc., San Francisco, CA, USA). The protein was transferred onto a polyvinylidene fluoride blotting membrane using a TE series transfer electrophoresis unit (Hoefer, Pharmacia Biotech Inc., San Francisco, CA, USA). The membrane was incubated in a blocking buffer for 1 hour and then incubated with polyclonal iNOS antibody (1:1000 dilution; Transduction Laboratories, San Diego CA, USA) overnight at 4°C. The membrane was washed and then incubated with a 1:2000 dilution of secondary antibody goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Pharmacia Biotech Inc., Buckinghamshire, England) at room temperature for 2 hours. The iNOS protein was detected using an ECL Western Blotting detection kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, England).

The Western blot procedures used for nitrotyrosine and COX-2 were similar to that described for iNOS. Instead of using the antibody against iNOS, liver homogenates were incubated with the anti-nitrotyrosine antibody (Upstate Biotechnology, Lake, Placid, NY, USA) and COX-2 (Cayman Chemicals, Ann Arbor, MI, USA) at 1:1000 for Western Blotting.

Measurement of NF-κB by electrophorectic mobility shift assay (EMSA)

Nuclear protein fractions from liver homogenates were prepared as described previously (Nanji et al., 1999). Electrophoretic mobility shift assays were performed on the nuclear extracts from livers in the different experimental groups as described previously (Nanji et al., 1999). Densitometric scanning analysis was performed using laser scanning densitometry. The specificity of binding was determined by prior addition of 100-fold excess of unlabeled competitor consensus oligonucleotide. Supershift experiments were performed on 5% non-denaturing gels using antiserum against the p50 subunit of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis

Data from each group were expressed as mean ± standard error of mean (SEM). Statistical comparison between two groups was done by using a non-parametric Mann Whitney U-test (two-tailed) using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). A P value of <0.05 was considered to be statistically significant.

Results

Weight of Control and NAFLD

There was a gradual increase in body weight in both control and NAFLD groups. No significant difference was observed between the two treatment groups (Fig. 1A).

Effect of high-fat diet on serum alanine aminotransferase (ALT) levels

The activity of serum alanine aminotransferase increased significantly by 2.5-fold in the NAFLD group when compared with the CF group. The ALT level of the CF group was at the level of about 70 U/L (Fig. 1B).

Effect of diet on collagen

The amount of collagen accumulation in the liver was visualized by Sirius Red staining in both groups. A higher percentage of collagen deposition was detected in the liver sections of NAFLD rats with a thickening of the connective tissue component around the central veins and the presence of pericellular and bridging fibrosis (Fig. 2C, D). There was a three-fold increase in the percentage area of collagen in the NAFLD rats when compared with the CF rats (Fig. 1C).

Effect of diet on histological observation

The NAFLD rats developed a greater degree of steatosis (Fig. 2). Extensive cytoplasmic fat deposition vacuoles were observed in the liver sections of the NAFLD group (Fig. 2B). Rats fed with regular chow showed a very minimal amount of fat deposition (Fig. 2A). Significant amounts of inflammation and necrosis were detected in the NAFLD group when compared to the CF group. Both fat deposition and necrosis increased about three-fold in the NAFLD group when compared to the CF group. The inflammatory score was also increased by about four-fold in the NAFLD group when compared to the CF group (Figs 2E-G).

Effects of diet on levels of adiponectin and anti-oxidant enzymes

The mRNA expression level of adiponectin, gluthatione peroxidase (GPX) and catalase (CAT) were significantly reduced in NAFLD rats with reductions of approximately 60%, 33% and 45% in comparison to the control group, respectively (Figs. 4A, 2I,J,H). The level of superoxide dismutase (SOD) was similar in the two groups.

Effects of diet on levels of pro-inflammatory mediators –TNF-α, iNOS and COX-2

The mRNA level of TNF-α was elevated by approximately five-fold in the NAFLD group compared with the CF group (Fig. 3C). The mRNA levels of both iNOS and COX-2 were increased about three-fold-and four-fold respectively in the NAFLD group when compared to the CF group (Fig. 3A,B). Similar trends were observed in the protein levels of both iNOS and COX-2 (Fig. 3D,E).

Effect of diet on a marker of oxidative stress (nitrotyrosine protein)

The level of nitrotyrosine, a product of nitric oxide and superoxide interaction, in the NAFLD rats showed a two-fold increase in nitrotyrosine formation when

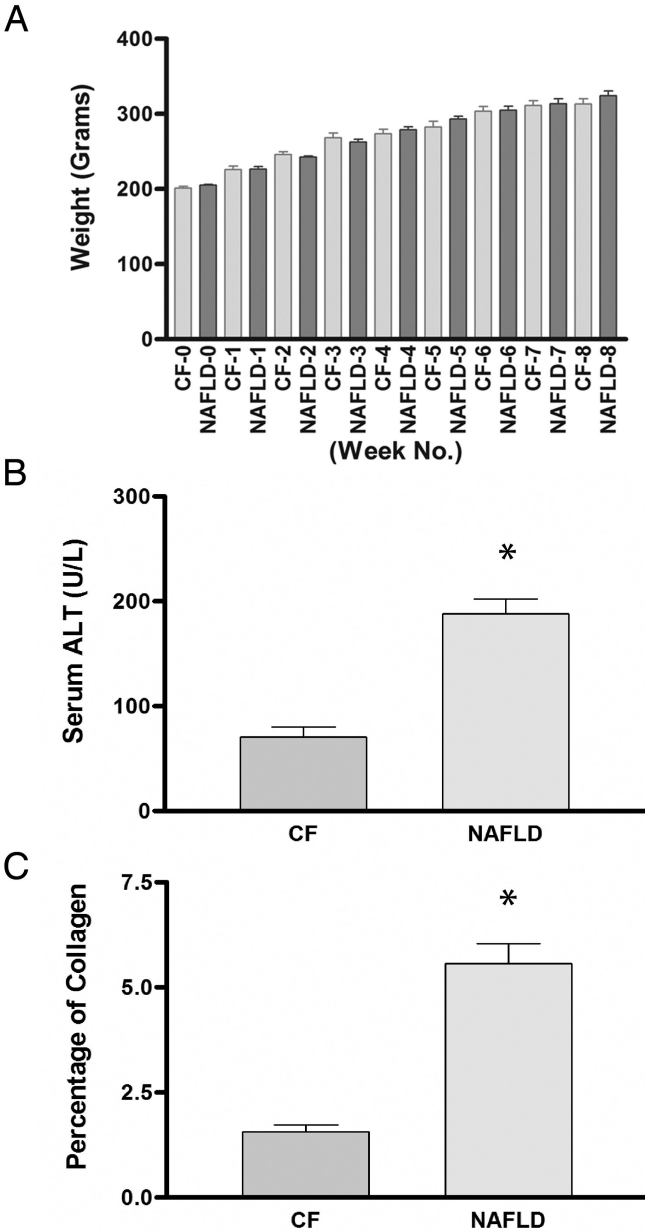
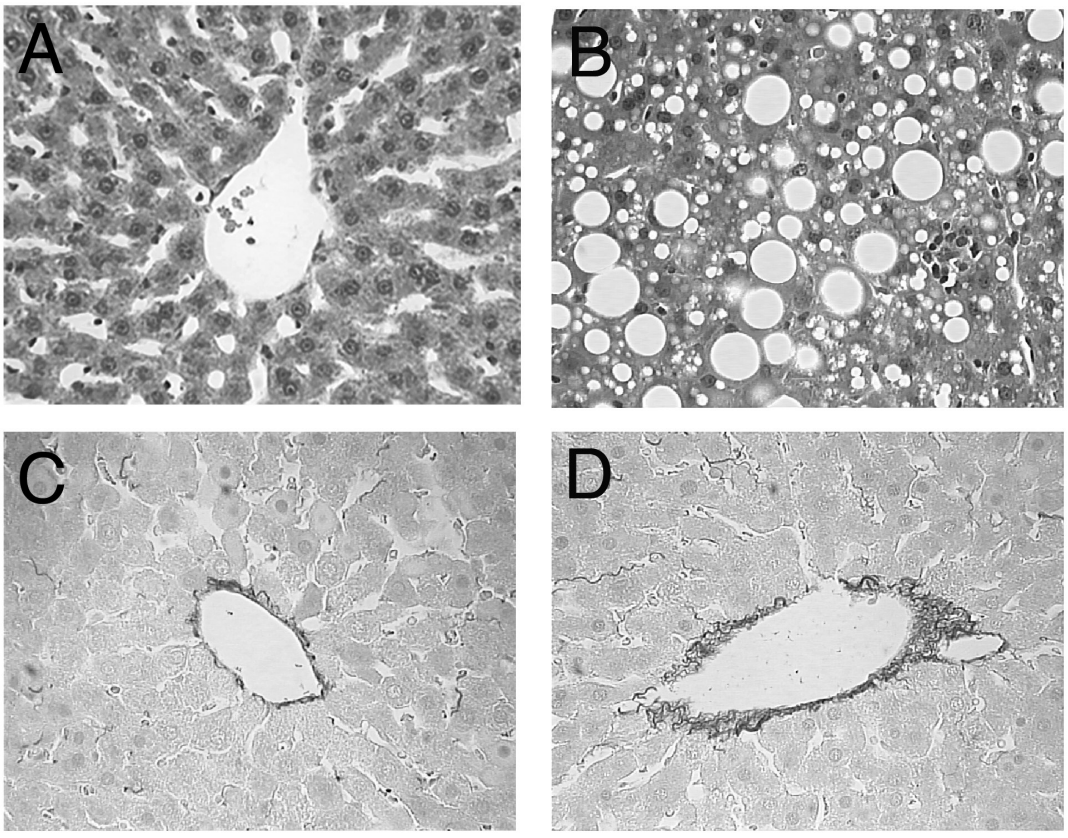


Fig. 1. A. Body weight of control (CF) and experimental rats (NAFLD). A gradual weight gain in both groups was observed. There was no difference in weights between the groups. **B, C.** Rats in the NAFLD group had a significantly higher level of serum ALT and accumulation of collagen in the liver than the CF group. *p-value <0.05



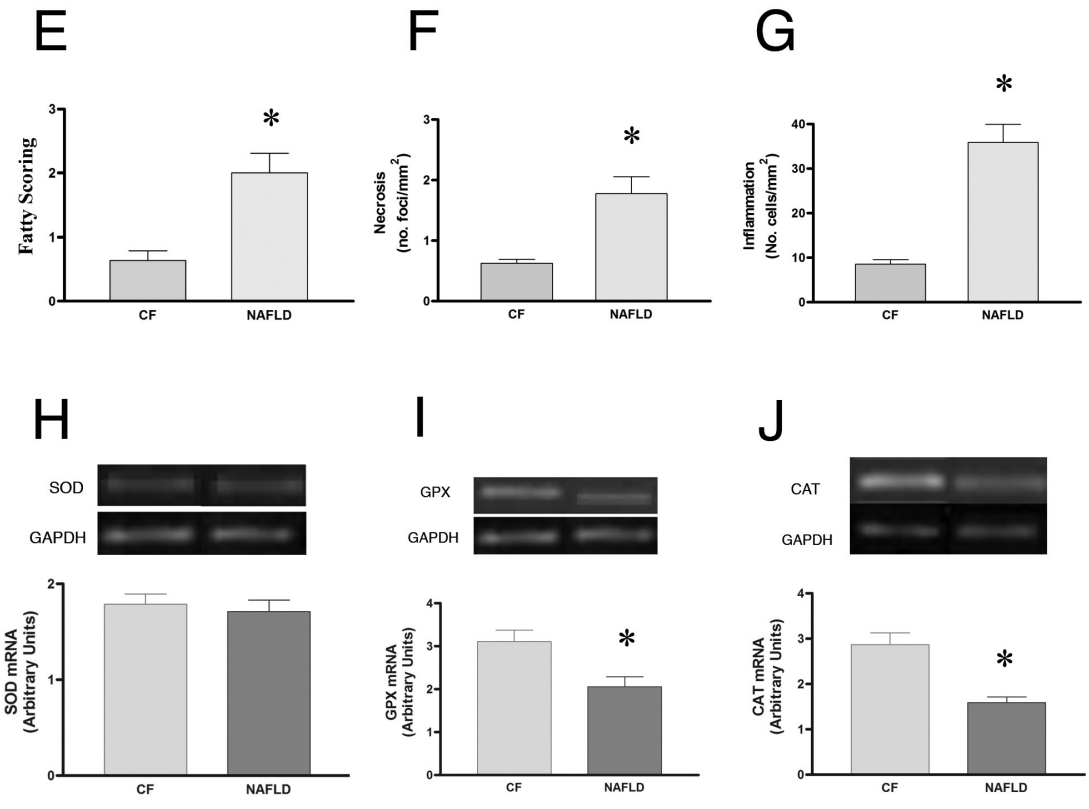


Fig. 2. Liver histology of rats. H&E staining of liver sections of **(A)** control and **(B)** NAFLD groups. Steatosis, formation of macrovesicular fat droplets and ballooning degeneration were observed in NAFLD liver section. Sirius Red staining of liver sections of **(C)** control and **(D)** NAFLD rats. A greater amount of deposition of collagen along the central vein is seen in NAFLD group. NAFLD group showed a greater degree of **(E)** fatty liver, **(F)** necrosis and **(G)** inflammation on liver samples. No difference in the mRNA expression level of **(H)** superoxide dismutase (Cu/Zn SOD) was noticed but a lower mRNA expression level of **(I)** glutathione peroxidase (GPx) and **(J)** catalase (CAT) was detected in NAFLD rats. *p-value <0.05. x 200

compared with the CF group (Fig. 3F).

Effect of high-fat diet on transcription factor NF-κB

To evaluate the activation of NF-κB, electrophoretic mobility shift assays of nuclear extracts from whole liver were performed. The degree of NF-κB activation significantly increased by approximately 8–fold in the NAFLD group when compared with the CF rats (Fig. 4B). The protein/DNA complex was further characterized by using competition and supershift assays. A 100-fold excess of non-radioactive NF-κB or STAT 3 oligonucleotide was added to an EMSA binding reaction containing nuclear protein extracts from the NAFLD and control rats. Addition of the NF-κB oligonucleotide completely abrogated complex

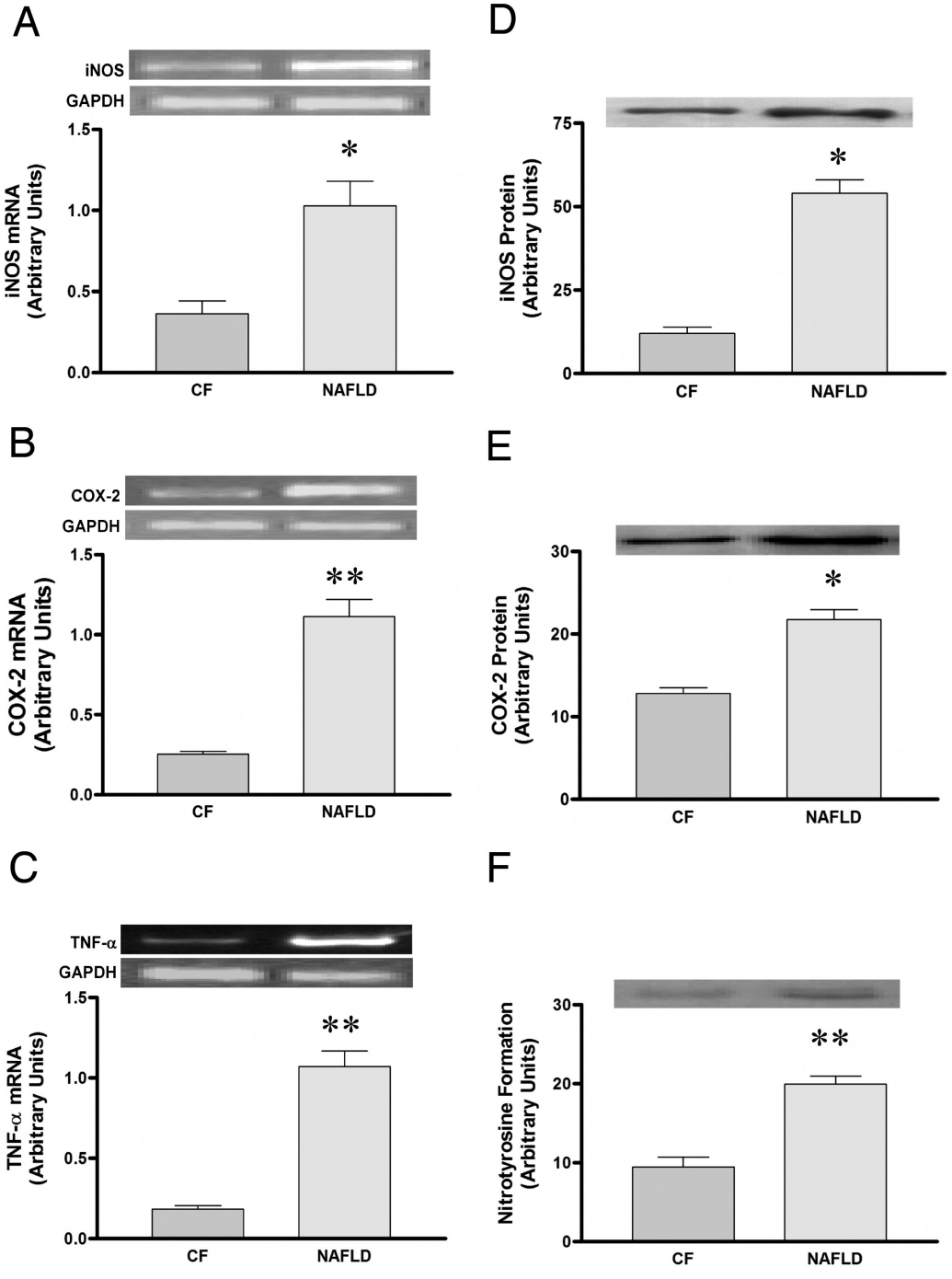


Fig. 3. Expression level of pro-inflammatory mediators. Rats fed with experimental fat diet showed a significant increase when compared to control in both mRNA and protein levels of iNOS **(A, D)**, COX-2 **(B, E)** and TNF-α. **(C)**. **(F)** NAFLD group also showed a significant upregulation in the formation of nitrotyrosine. *p-value <0.05; **p-value <0.01.

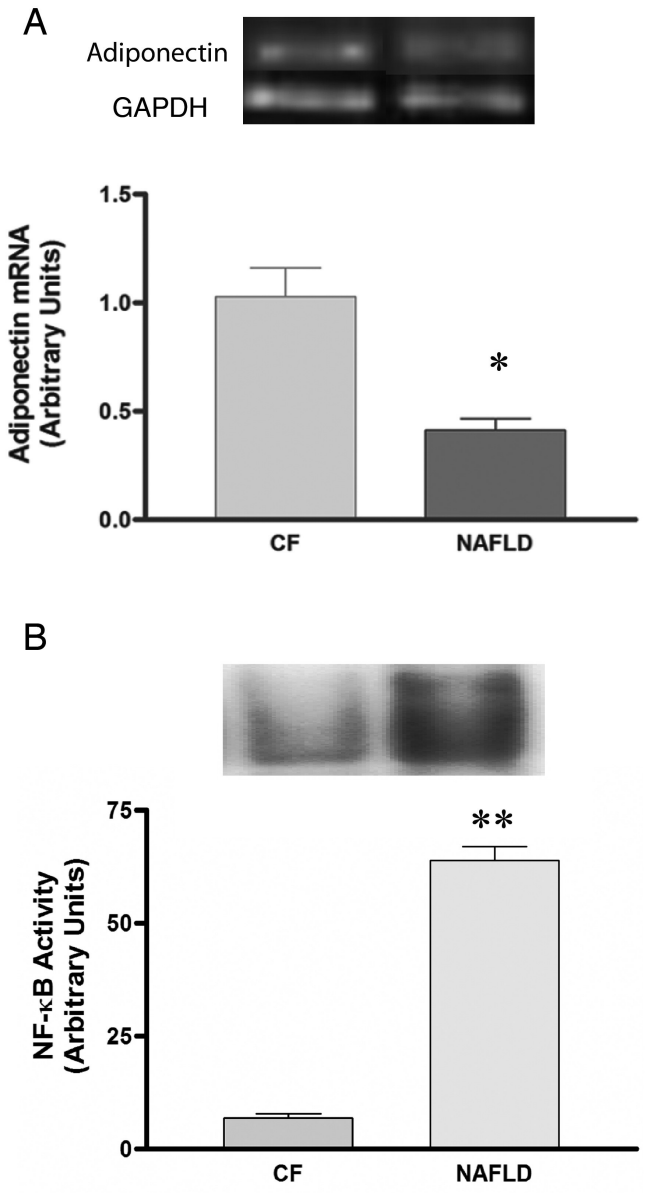


Fig. 4. A. Rats fed with the experimental diet (NAFLD) showed a reduction in adiponectin mRNA levels when compared with the controls. **B.** The DNA-binding activity of the transcription factor, NF-κB, was significantly elevated in the NAFLD group over the control group. *p-value <0.05 ; **p-value <0.01

formation, and addition of STAT 3 oligonucleotide had no effect (data not shown).

Discussion

Animal Model of NAFLD

The prevalence and clinical studies of non-alcoholic fatty liver diseases (NAFLD) are informative but fail to provide relevant information concerning the disease progression and experience difficulty in evaluating in detail the therapeutic values of individual treatment. Many studies used animal models with genetic defects which render them extremely vulnerable to environmental factors or used a diet that is incomplete. Dietary models of NAFLD are commonly used but require excess calories (Nanji, 2004b). For example, Lieber et al., 2004 used a diet which was extremely high in fat (>70%) and successfully reproduced the key features of non-alcoholic fatty liver disease (NAFLD) in a rat model. This high fat diet induced steatosis, patchy inflammation, pronounced oxidative stress, defects in the mitochondria, increased plasma insulin concentrations, and raised hepatic concentrations of tumor necrosis factor-α and collagen. We used in the present study a diet that contained dextrose, fat (fish oil), protein plus salt mix, vitamins, choline betratrate and methionine, which were more physiological in content. Another major advantage was that we did not use the gavage or intragastric feeding method, which is expensive and technically demanding (Zou et al., 2006).

Our group has previously shown the role played by unsaturated fat in the induction of alcoholic liver disease (Nanji, 2004a). We suggest that the amount and quality of unsaturated fat may also be an important factor in inducing NAFLD (Cave et al., 2007; Elizondo et al., 2007; Machado et al., 2007). To test this hypothesis, we developed a rat model that consumed a highly unsaturated fat as the main source of fat. The animal model showed hepatic steatosis, inflammation, necrosis, ballooning degeneration and evidence of collagen accumulation. We also found that there is a 2.5 fold increase in the serum ALT levels in the NAFLD rats.

We know that ad libitum consumption of diet can be self-limiting, either because of diet composition or palatability (Corbertt et al.,1986; Maegawa et al., 1986; Watarai et al., 1988; Lieber et al., 2004). Our present model is a preferred model for two reasons. Firstly, we observed (as shown in Fig. 1) that the increase in the body mass of the NAFLD rats resembles the clinical situation, with excessive intake of calories and resulting in obesity. In fact, our results additionally show that considerable liver damage can occur without any observed signs of obesity. Secondly, as reviewed by Food and Agriculture Organization of the United Nations (FAO), the Fat Energy Ratio i.e. FER which is the proportion of dietary energy derived from total fat, ranges from 7-46% (FAO Food Nutr Pap., 1994). The model used in this study requires only 30% fat, which is closer to the usual intake. Thus, we have been able to reproduce the principle pathological features of NAFLD using a voluntary oral feeding method and without the use of a very high-fat diet regimen.

Histological findings of steatosis, necroinflammation and collagen deposition

The most widely accepted explanation for the pathogenesis of NAFLD favors multiple hit hypothesis (Day and James, 1998; McCullough, 2006). The first hit is the development of steatosis. Histological findings in

the present study confirmed the accumulation of lipids within the hepatocytes of the NAFLD rats.

We also observed numerous necrotic foci with increased infiltration of inflammatory cells in the NAFLD rats. According to the "multiple-hit" hypothesis, the second and subsequent hits lead to progression from steatosis to steatohepatitis. It has been previously hypothesized that inflammatory cytokines, lipid peroxidation and oxidative stress are some of the main underlying events that contribute to the development of NAFLD (McCullough, 2006). The results of the present study showed that nitrotyrosine formation, a product of nitric oxide and superoxide, was significantly increased in the NAFLD rats. This finding was accompanied by a significant increase of iNOS levels at the transcriptional and translational levels. Overproduction of NO by iNOS results in peroxynitrite formation in the NAFLD rats (Koppenol, 1998). Studies have shown that NO becomes a more potent oxidant when combined with superoxides to form peroxynitrite, which causes direct cell injury (Pessayre et al., 2001; Glantzounis et al., 2005).

In the present study, a three-fold increase in the amount of collagen deposition around the central veins and the perihepatic regions in the NAFLD group indicated that hepatic stellate cells (HSC) were activated. HSC activation occurs in patients with NAFLD (George et al., 2003; Feldstein et al., 2005).

Activation of NF-κB and induction of innate immunity response

In the present study, the levels of both NF-κB and TNF-ß were upregulated in the NAFLD rats, which represent key molecules in the inflammatory responses. NF-κB is a sequence specific transcription factor that functions as a proinflammatory "master switch" during inflammation and upregulation of the transcription of a wide range of inflammatory mediators.

TNF-α is involved in apoptotic cell death, proliferation and inflammation and its expression level was increased in the NAFLD rats. This increase in TNF-α level concurs with the histological findings in the liver of NAFLD rats. The upregulation of TNF-α then stimulates the Kupffer cells and other liver cells to produce various proinflammatory mediators and reactive oxygen species that further induce cellular inflammation and injury (Galun and Axelrod, 2002; Hu, 2003). Other proinflammatory mediators that are involved in the induction of inflammation include iNOS and COX-2. In the present study, both mRNA and protein levels of iNOS and COX-2 were upregulated in the NAFLD group.

Reduced Adiponectin in NAFLD

In the current experiment, rat hepatic concentration of adiponectin was decreased by 60% in the NAFLD group. Adiponectin is an adipocytokine produced by adipose tissue that opposes the effects of systemic insulin resistance in liver lipid partitioning. The observed reduction of hepatic adiponectin level in the NAFLD group suggests that reduction in fatty acid beta-oxidation and increased synthesis contributes to fatty liver. Adiponectin also exerts an anti-inflammatory effect by suppressing the release of TNF-α from macrophages within adipose tissue (Masaki et al., 2004). Therefore, the low level of adiponectin promotes both fatty liver and an increase in the level of TNF-· expression in the livers of NAFLD rats.

Imbalance of Pro-oxidant and Anti-oxidant Enzymes

In the present study, the levels of catalase (CAT) and glutathione peroxidase (GPx) were reduced significantly in the NAFLD rats. Consistent with other studies, our findings confirm that an increase in oxidative stress and a suppression of antioxidant capacity play an important role in the development and progression of NASH and NAFLD (Cortez-Pinto et al., 2001; Pessayre et al., 2002; Leclercq, 2004; Videla et al., 2004). Superoxide dismutase (Cu/Zn SOD) levels were unchanged in both NAFLD and CF rats, suggesting that the superoxide scavenging capacity was not affected. It is important to note that SOD, CAT and GPx have complementary roles in antioxidant defense.

In conclusion, a clinically relevant animal model was established that involved rats fed with a 30% fat diet. This is a more physiological model than previously used models, which used an extremely high fat content in the diet. Furthermore, the rats ate voluntarily. This animal model showed evidence of increased fat deposition in the liver, necroinflammation, collagen deposition, activation of proinflammatory mediators and reduced adiponectin, GPx and CAT activity. We proposed that this animal model could be a useful tool to investigate the pathogenesis of NAFLD and may provide a platform for testing new therapeutic agents.

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