Rapid Development of Non-Alcoholic Steatohepatitis in Psammomys obesus (Israeli Sand Rat)

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Abstract

Background and Aims: A major impediment to establishing new treatments for non-alcoholic steatohepatitis is the lack of suitable animal models that accurately mimic the biochemical and metabolic characteristics of the disease. The aim of this study was to explore a unique polygenic animal model of metabolic disease as a model of non-alcoholic steatohepatitis by determining the effects of 2% dietary cholesterol supplementation on metabolic and liver endpoints in Psammomys obesus (Israeli sand rat).

Methods: P. obesus were provided ad libitum access to either a standard rodent diet (20% kcal/fat) or a standard rodent diet supplemented with 2% cholesterol (w/w) for 4 weeks. Histological sections of liver from animals on both diets were examined for key features of non-alcoholic steatohepatitis. The expression levels of key genes involved in hepatic lipid metabolism were measured by real-time PCR.

Results: P. obesus fed a cholesterol-supplemented diet exhibited profound hepatomegaly and steatosis, and higher plasma transaminase levels. Histological analysis identified extensive steatosis, inflammation, hepatocyte injury and fibrosis. Hepatic gene expression profiling revealed decreased expression of genes involved in delivery and uptake of lipids, and fatty acid triglyceride synthesis, and increased expression of genes involved in very low density lipoprotein cholesterol synthesis, triglyceride and cholesterol export.

Conclusions: P. obesus rapidly develop non-alcoholic steatohepatitis when fed a cholesterol-supplemented diet that appears to be histologically and mechanistically similar to patients.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is the world’s most common form of chronic liver disease, and affects approximately 75% of patients with obesity and type 2 diabetes [1]. The presence of NAFLD worsens outcomes in these patients, as it exacerbates diabetes, and can lead to cirrhosis and liver cancer [2]. It is estimated that approximately 20% of NAFLD patients will go on to develop non-alcoholic steatohepatitis (NASH), and approximately 20% of NASH patients will progress to life-threatening cirrhosis [3]. The presence of NASH in human patients is confirmed by key histological features including steatosis, inflammation, hepatocyte injury, and fibrosis [4,5].

Current management of NASH generally focuses on weight loss and treatment of co-morbidities (such as insulin resistance [6]). A major impediment to establishing and characterising new treatments for NASH is the lack of suitable animal models that accurately mimic the biochemical and metabolic characteristics of NASH in human patients [7]. Current animal models of NAFLD or NASH can be broadly categorised as either dietary-induced or genetic. The most widely used dietary-based model is rodents fed a methionine- and choline-deficient (MCD) diet high in sucrose and fat, resulting in the prevention of hepatic β-oxidation and impaired production of very low density lipoprotein (VLDL). This leads to the accumulation of intrahepatic lipid, fibrosis and decreased VLDL synthesis. However, the MCD diet is associated with considerable weight loss and toxicity [7,8]. Additionally, the metabolic profile of animals fed this diet includes decreased plasma insulin and glucose levels, in contrast to the metabolic profile observed in the majority of human patients with NAFLD/NASH [7,8]. Db/db mice, which have a mutated leptin receptor gene, are a widely used genetic model of NAFLD. These animals develop obesity, insulin resistance, diabetes and hepatic steatosis, however
only progress to NASH following additional intervention such as exposure to the MCD diet [9]. Although some of these animal models are useful for investigation into particular aspects of human NAFLD/NASH, none display all of the histological characteristics present in the human disease state and in the context of metabolic disease.

Psammomys obesus (Israeli sand rat) is a gerbil that is a unique animal model of the Metabolic Syndrome. In its native desert environment, P. obesus remain lean and healthy. However, when housed under laboratory conditions and fed a standard rodent diet (20% kcal/fat), a proportion of P. obesus become obese and type 2 diabetic, while others remain lean and healthy [10–14]. Previous studies have shown P. obesus to be susceptible to steatosis when fed a standard rodent diet with added fibre (30% wheat straw), however key features of human NASH such as fibrosis, hepatocyte injury and inflammation were absent [15]. The majority of previously reported rodent models of NAFLD/NASH utilize diets high in fat and/or supplemented with cholesterol. As P. obesus will not consume highly palatable energy-rich diets, we tested whether NASH could be induced in P. obesus via addition of cholesterol (2% w/w) to their standard rodent diet (20% kcal/fat). In this study we show that dietary cholesterol supplementation for 4 weeks induces NASH in P. obesus with a profile similar to that seen in patients.

Materials and Methods

Ethics Statement

Animals were maintained in accordance with the Code of Practice of the National Health and Medical Research Council of Australia, and all procedures were carried out subject to the approval of the Deakin University Animal Ethics Committee (permit number A59-2010). All efforts were made to minimise animal suffering.

Experimental animals

A colony of outbred P. obesus is maintained at Deakin University, Geelong, Australia. All animals were housed in a temperature-controlled room (22 ± 1°C) with a 12–12 h light-dark cycle (light 06:00–18:00 h).

Male animals aged 8–10 weeks were randomly allocated to the following groups: 1) Standard diet (20% kcal/fat, 0% cholesterol; n = 8), or 2) Cholesterol-supplemented diet (20% kcal/fat, 2% cholesterol/w/w; n = 9). Both diets contained 16% kcal/protein and 64% kcal/carbohydrate, and had total digestible energy of 15 MJ/kg. The source of fat for both diets was mixed vegetable oils. There was no difference in % fatty acid profile or total calories.

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Histological and biochemical analyses of liver tissue

Animals were humanely killed and 1 g of liver tissue (left lobe) was removed and fixed in 10% neutral-buffered formalin. The liver tissue was paraffin-embedded, sectioned (5 μM), mounted and stained with hematoxylin and eosin, or Masson trichrome stain (Sigma-Aldrich, St Louis, MO) to visualise fibrosis. All histological analyses were conducted by a pathologist blinded to the treatment conditions.

Pictures of Masson trichrome stained liver sections were taken on an Axioskop 2 microscope (Zeiss; magnification 200x) and collagen content was quantitated using ImageJ software (NIH). For each animal, 10 fields per liver section were selected randomly and blue staining (representing collagen) was highlighted. The area of staining was measured as number of pixels per picture and the data collated to give a mean area of staining per field for each animal in the study.

An additional portion of the liver (right lobe) was used to measure lipid content. Lipid was extracted using a modified Folch protocol [16]. The tissue was homogenised in 2:1 chloroform/methanol solution (10 ml), and filtered. An additional 5 ml of 2:1 chloroform/methanol solution was added, followed by 2.5 ml of 0.9% NaCl. After mixing, the extract was centrifuged for 5 min at 2 000 g at 10°C. After discarding the aqueous layer, the organic layer was dried under nitrogen, and total lipid content assessed by weighing.

To assess the types of lipid present, a 1 g portion of the liver (right lobe) was minced and lipids extracted essentially as previously described [17]. The lipid extract was reconstituted in 200 μl of dichloromethane and the lipids were then separated by thin layer chromatography. The lipid extracts were spotted onto silica gel plates (Silica gel 60 G, Merck, Germany) and developed in 85:15:2 (v/v) petroleum ether: diethylether: acetic acid in paper-lined tanks. The lipids were visualised under ultraviolet light after reacting with 0.1% (w/v) 2',7’-dichlorofluorescein indicator in ethanol (Scharlau, Spain).

Plasma enzyme and metabolite analyses

Blood glucose was measured on days 0 and 28 using a glucometer (Accuchek II; Roche, Castle Hill, Australia). Plasma triglyceride, total cholesterol, HDL-cholesterol, ALT and AST levels were measured using an Olympus AU400e Bioanalyzer (Olympus America Diagnostics, Center Valley, PA). Plasma insulin concentration was measured using an ultra-sensitive ELISA kit (Crystal Chem Inc, Downers Grove, IL, USA).

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from 100 mg of liver tissue using TRIzol (Invitrogen Life Technologies, Carlsbad, USA) and RNeasy columns (Qiagen, Hilden, Germany). Quality and concentration of mRNA was established using RNA 6000 Nano Assay on a Bioanalyzer (Agilent Technologies, USA), and cDNA was generated from total RNA using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Gene expression levels were measured using FastStart Universal SYBR Green Master (Roche Australia) on a Mx3005P cycler (Stratagene), and RT-PCR primer sequences are listed in Table 1.

Statistical Analysis

Data distribution was tested for normality using a Kolmogorov-Smirnov test. Group mean differences were assessed using Student’s unpaired t-test (for normally distributed data) or Mann-Whitney U-test. Correlation between continuous variables was assessed using Pearson (for normally distributed data) or Spearman rho tests as appropriate. All analyses were performed using Statistical Package for the Social Sciences software (SPSS version 20; USA). Data were considered statistically significant at p<0.05.

Results

We evaluated the effects of feeding lean, non-diabetic P. obesus a standard rodent diet (20% kcal/fat) supplemented with 2% cholesterol for 4 weeks. There was no difference in food intake between the groups (standard diet 52±3 v. cholesterol-supplemented diet 47±3 g/kg/d, p = 0.26). The mean body weight of the two groups was not different at the start or end of the study,
Liver sections from animals on both diets were examined for histological features of NASH (Fig. 3). Blinded histopathological analysis confirmed the presence of steatosis, inflammation, hepatocyte injury and fibrosis in the cholesterol-supplemented group. Steatosis was marked, with abundant large and small lipid droplets (Fig. 3D.). Inflammation was demonstrated by the presence of neutrophil-containing parenchymal inflammatory foci (Fig. 3C.), and in addition sinusoidal foamy macrophages were seen in some livers (Fig. 3B.). This was supported by greater mRNA expression of proinflammatory markers including CD68, interleukin 1 beta (IL1B), chemokine (C-C motif) ligand 2 (CCL2) and eff-like module containing, mucin-like, hormone receptor-like 1 (EMR1) (Fig. 4, p<0.05 for each gene). Hepatocyte injury was noted, including focal necrosis in some cases, and there was architectural disruption including fibrous expansion of portal tracts, early septum formation, and both pericellular and perisinusoidal fibrosis (Fig. 3D,E,F.). This fibrosis was reflected by markedly higher collagen 1 alpha (COL1A1), actin, alpha 2, smooth muscle, aorta (ACTA2) and transforming growth factor, beta 1 (TGFB1) gene expression (Fig. 4, p<0.05 for each gene). Collagen content was 30-fold increased in the cholesterol-supplemented group compared with controls as assessed by quantitative imaging (11756±5859 vs. 378±224 pixels per field, p<0.002).

NAFLD activity scores were calculated based on the method of Kleiner and Brunt [18]. The overall scores were 4.4±0.2 in the cholesterol-supplemented group and 1.9±0.4 in the control group (p<0.001). Scores were significantly different for steatosis (2.3±0.3 vs. 1.3±0.2, p=0.007) and inflammation (2.1±0.4 vs. 0.5±0.3, p=0.003). Histologically, P. obesus showed the key features of NASH after only 4 weeks of dietary cholesterol supplementation. The effect on the liver by simply adding cholesterol to the diet (without increasing dietary fat content) was profound in P. obesus compared with effects seen previously in other rodents, suggesting increased susceptibility to NASH in these animals. Therefore, we profiled the hepatic expression of key genes involved in lipid metabolism in P. obesus fed the two diets. Expression of genes involved in delivery and uptake of lipids were consistently lower in the cholesterol-supplemented group (SCARB1 -37%, p = 0.019; SCARB1 -48%, p = 0.002; LDLR -61%, p <0.001; PCSK9 -50%, p = 0.022; Table 2), while genes involved in fatty acid and triglyceride synthesis were also lower in the cholesterol-supplemented group (FABP1 -48%, p = 0.012; PPARA -59%, p<0.001; GPAM -41%, p = 0.016; LIPE -49%, p = 0.005; Table 2). In general, genes encoding proteins involved in VLDL synthesis, triglyceride and cholesterol export from the cells were higher in the cholesterol-supplemented group (ACAT2 +63%, p = 0.034; ABCA1 +230%, p = 0.001; Table 2), however expression of MTTP was unexpectedly lower (-43%, p=0.007). PPARA gene expression was increased (+170%, p = 0.007), presumably to increase transcription of genes involved in fatty acid oxidation, however CPT1A mRNA was decreased (-41%, p = 0.027).

Finally, the expression of cholesterol 7 alpha hydroxylase (CYP7A1), the rate-limiting step in bile acid synthesis, was reduced in livers of P. obesus fed the cholesterol-supplemented diet (-47%, p=0.016).

**Discussion**

Here we have shown that P. obesus exhibit key features of NASH when exposed to a cholesterol-supplemented diet. Four weeks of dietary cholesterol supplementation resulted in these animals displaying extensive steatosis, inflammation, hepatocyte injury and fibrosis. These histological phenotypes closely resemble the NASH disease profile observed in human patients. Additionally, hepatic

| Gene     | Forward primer (5'-3') | Reverse primer (5'-3') |
|----------|------------------------|------------------------|
| CD68     | ggacagtaccttctccggctaa | cttgaggaattaacttgcattc |
| IL18     | atcagctcctttcttccaagc | agagagttctttactagaacg |
| CCL2     | ccagcttctctctcttctctctc | ccagcttctctctcttctctc |
| ENM1     | gcaatgtctgtcagccttctt | gcatacttgagtgcctgcttg |
| COL1A1   | cgtggaagctggtgagcttgc | ggtgagatgtggtctgag |
| ACTA2    | tcgggtgtggtggttgg gcgtgagatgtgttgctgagg |
| TGFB1    | aaaggaggacagtgtttc gatgaggttggagatagc |
| SREBF2   | tctccagagggagagagctcag | caggggctgccatctgtgctag |
| SCARB1   | gtctacaggaggtcagaca | taggcaagctacaattgtagcag |
| LDLR     | tgcgggtggtcggcttagact | cccactcttgagatcagacc |
| PCSK9    | ggttctctctctctctctctc | cccactcttgagatcagacc |
| FABP1    | gccatatgacccctccggct | ctggatcctctacctctgtctgagt |
| PPARA    | tgatcattgctgagacctgt | cagacatcctgcttcctcttc |
| GPAM     | gatcagcagagagagctcag | cagacagcagagagctcag |
| HSL      | cggagacaggcccctggtga | aacctggtctttgagcgaact |
| LIPE     | tccgggtgggtgggtgggtg | gctgactgtggtctgag |
| MTTP     | gttctctctctctctctctc | cccactcttgagatcagacc |
| ACAT2    | tagcactttcactccaaaac | cccaggtcgctctcttccttc |
| ABCA1    | aaaggagacagtcttctt | gcatacttgagtgcctgcttg |
| PPARA    | ggtcgccctgcgtgaggtgagagcttgc | ggtcgccctgcgtgaggtgagagcttgc |
| CPT1A    | tgacgagatcactgacagacc | agccatcagcagagagctcag |
| CYP7A1   | tctagagagggagagagctcag | aagagagggagagagctcag |
| Cyclophilin | cccagtcgtggtcttcctcagaca | ccagccagtgcagcagcagaaa |

**Table 1. PCR primer sequences.**

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gene expression data confirmed that the NASH in *P. obesus* appears to be mechanistically similar to that in patients with NASH.

Brunt and colleagues [4,5] have established criteria for the histological diagnosis of NASH. The key features are steatosis, hepatocyte injury and inflammation, typically localised in zone 3, with or without fibrosis. In this study we have shown the presence of all of these key features of NASH in livers of *P. obesus* fed a cholesterol-supplemented diet for 4 weeks. The steatosis was closely associated with foci of inflammation, and was particularly evident in hepatic zone 3. Our evidence for hepatocyte injury were less clear, although the histological appearance suggested significant disruption of the cellular architecture, and this was supported by increased plasma levels of transaminases that are known to be released from injured hepatocytes. Furthermore, patches of necrosis observed in two of the animals also support the suggestion of hepatocyte injury, even though necrosis is not a common feature of human NASH [4,5]. Overall, the histological appearance of NASH in *P. obesus* closely resembles that seen in human patients.

In contrast to other dietary based animal models, the diet used in this study involved only a minor modification to a standard rodent diet. Other dietary models are either based on nutrient deficiency (e.g. MCD diet) or unrealistically high energy diet (e.g. 60–80% kcal/fat, 80% fructose) [7,8]. In this study we added 2% cholesterol to a standard rodent diet, from which 20% of energy was derived from fat, which is not considered to be a high fat diet. The addition of 2% cholesterol to the diet can be considered relatively high when compared with previous studies using 0.5–1% dietary cholesterol [19,20]. However, more recently, 2% dietary cholesterol in the context of high fat, high fructose diet (where the
source of fat was trans-fat elicited fibrosis in leptin-deficient mice, but not in normal C57BL6 mice [21], suggesting that multiple insults are required in other rodent models for the full spectrum of NASH, not just increased dietary cholesterol [22]. The presence of significant hepatic damage induced by relatively minor dietary variation suggests that *P. obesus* may be an attractive model for future NASH research compared with other dietary-based models.

Figure 3. Histopathology of NASH in *P. obesus*. Masson’s trichrome stained livers at ×400 magnification except F (×40). A: Control group with minimal steatosis and no fibrosis, B: Lipid droplets in most cells, and sinusoidal foamy macrophages (arrows), C: Parenchymal inflammation with neutrophils (arrow), D: Strands of pericellular and perisinusoidal collagen (arrows), E: Fibrous expansion of portal tracts, with oval cells/ductular reaction, F: Distorted architecture, with portal septum formation.

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Figure 4. Hepatic gene expression in *P. obesus* with NASH. Gene expression of markers of inflammation and fibrosis in livers of *P. obesus* with NASH, n = 8–9 per group. *p<0.05, **p<0.005.

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Table 2. Gene expression in livers of P. obesus fed the cholesterol-supplemented diet, expressed as fold difference relative to animals fed the standard diet.

| Gene Name                        | Gene Symbol | Fold difference | P    |
|----------------------------------|-------------|----------------|------|
| Delivery and uptake of lipids    |             |                |      |
| Sterol regulatory element binding transcription factor 2 | SREBF2      | 0.63±0.07      | 0.019|
| Scavenger receptor class B, member 1 | SCARB1      | 0.52±0.08      | 0.002|
| Low density lipoprotein receptor | LDLR        | 0.39±0.04      | <0.001|
| Lipoprotein convertase subtilisin/kexin type 9 | PCSK9       | 0.58±0.12      | 0.022|
| Lipogenesis                      |             |                |      |
| Fatty acid binding protein 1, liver | FABP1       | 0.52±0.09      | 0.012|
| Peroxisome proliferator-activated receptor alpha | PPARA       | 0.41±0.08      | <0.001|
| Glycerol-3-phosphate acyltransferase, mitochondrial | GPAM        | 0.59±0.09      | 0.016|
| Hormone-sensitive lipase         | LIPE        | 0.51±0.05      | 0.005|
| Fatty acid synthase              | FASN        | 0.72±0.06      | 0.18 |
| Lipid export                     |             |                |      |
| Acetyl-CoA acetyltransferase 2   | ACAT2       | 1.63±0.24      | 0.034|
| ATP-binding cassette, sub-family A (ABC1), member 1 | ABCA1       | 3.30±0.49      | 0.001|
| Microsomal triglyceride transfer protein | MTTP        | 0.57±0.08      | 0.007|
| Lipid oxidation                  |             |                |      |
| Peroxisome proliferator-activated receptor delta | PPARD       | 2.70±0.18      | 0.007|
| Carnitine palmitoyltransferase 1A (liver) | CPT1A       | 0.59±0.12      | 0.027|
| Bile acid synthesis              |             |                |      |
| Cholesterol 7 alpha hydroxylase  | CYP7A1      | 0.53±0.10      | 0.016|

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In this study we have shown the development of NASH in P. obesus occurs after only four weeks. Such rapid onset is not observed in other dietary models with exposure to MCD diet requiring up to 10 weeks to lead to the development of NASH (depending on species/strain), whilst exposure to a high fat diet, fructose diet, or an atherogenic diet can take up to several months before NASH is observed [7,8]. We note that the animals in this study were not obese. It will be of interest to repeat this study in animals with pre-existing obesity and/or type 2 diabetes and investigate how this affects the development of NASH.

In P. obesus fed the cholesterol-supplemented diet for 4 weeks there was a consistent reduction in the liver mRNA levels of genes encoding proteins involved in the delivery and uptake of lipids. Of note, LDL receptor (LDLR) mRNA was 61% lower in animals fed the cholesterol-supplemented diet, which would be expected to result in reduced lipid and cholesterol uptake by the cells. This is opposite to what has been observed in mouse models of NASH [23], but consistent with observations in African Green Monkeys with NASH [24]. We also observed reductions in genes encoding proteins involved in de novo fatty acid and triglyceride synthesis. Notably, fatty acid binding protein 1 mRNA was significantly reduced in P. obesus with NASH, as it is in human cases [25], but not in mouse models of NASH [26]. Gene expression of PPARA and hormone sensitive lipase (LIPE) were reduced in P. obesus with NASH, as they are in humans with NASH [27,28].

Hepatic gene expression of ACAT2, which is involved in the removal of cholesterol from hepatocytes via VLDL, and ABCA1, which is involved in direct cholesterol efflux from hepatocytes, were both significantly increased in P. obesus with NASH. Increased ACAT2 gene expression is also a feature of human NASH [29]. Gene expression of MTTP was unexpectedly reduced, as has been seen in various mouse models of NASH [30,31]. CPT1A gene expression was also unexpectedly reduced, similar to what has been observed in human NASH [28,32], possibly due to excessive accumulation of malonyl-CoA in advanced NASH. Finally, the gene expression of cholesterol 7 alpha hydroxylase (CYP7A1), the rate limiting step in bile acid synthesis, was reduced by 47% (p = 0.02) in livers of P. obesus with NASH. This is counterintuitive, as bile acid synthesis is a major path for removal of cholesterol from the liver. However a similar result was also found in monkeys with NASH [24], and is thought to represent a measure to reduce intestinal cholesterol absorption by limiting bile acid availability, in an attempt to restore hepatic cholesterol homeostasis.

In summary we observed a general decrease in the expression of genes encoding proteins involved in the uptake and synthesis of fatty acids and triglycerides in the liver, and an increase in the expression of genes encoding proteins that function in the export or oxidation of lipids. Collectively these data indicate that the hepatic molecular characteristics of NASH in P. obesus resemble those seen in the human disease.

P. obesus rapidly develop NASH when fed a cholesterol-supplemented diet including marked steatosis, inflammation, hepatocyte injury and fibrosis. In general, the gene expression patterns and biochemical and histological features observed in P. obesus with NASH closely resembled the human condition. We conclude that P. obesus is a new animal model of NASH with significant advantages over existing models.

Author Contributions
Conceived and designed the experiments: KW JLT JDR. Performed the experiments: BS TC CW LA GK AC SM SL. Analyzed the data: BS AK MZ AS YG AEO RS KW. Contributed reagents/materials/analysis tools: AS JLT JDR AEO RS KW. Wrote the paper: BS RS KW.
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