Video Article

Incorporation of a Survivable Liver Biopsy Procedure in Mice to Assess Non-alcoholic Steatohepatitis (NASH) Resolution

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Abstract

Clinical trials assessing therapies for the treatment of non-alcoholic steatohepatitis (NASH) involve a baseline and end of study liver biopsy, and assessment of improvement in disease endpoints, often reflected as a percent of each treatment arm that improved, worsened or remained unchanged. Traditional preclinical rodent studies for putative NASH therapies are often limited by not knowing the level of liver disease/NASH present at the start of therapeutic intervention, instead of randomizing treatment groups on easily measurable endpoints such as body weight, metabolic status or similar. Here, we describe a liver biopsy technique in a diet-induced NASH mouse model, for the assessment of baseline liver disease in order to exclude mice that do not exhibit fibrosis and to equally distribute animals with similar fibrosis between treatment groups. These levels can then be compared to the terminal, post-intervention levels for a truer understanding of in vivo pharmacological effects and thus more accurately reflect clinical trial design strategies. The mouse is properly anesthetized and prepared for the surgery using sterile conditions. A small incision is made in the upper abdomen and the left lateral lobe of the liver is exposed. A wedge of the liver is surgically removed, and a similar-sized piece of absorbable gelatin is put in its place to stop any bleeding. The mouse is surgically sutured and stapled closed and will recover back to normal within 1 day. The entire process takes 5-10 min per mouse. Here we exemplify the utility of this procedure by leveraging the pre-study biopsy to assess the impact of the glucagon-like peptide-1 (GLP-1) receptor agonist liraglutide on NASH endpoints in mice.

Video Link

The video component of this article can be found at https://www.jove.com/video/59130/

Introduction

Non-alcoholic steatohepatitis (NASH) is a progressive liver disease, and a more severe form of nonalcoholic fatty liver disease (NAFLD), that is becoming increasingly prevalent due to its association with obesity and type 2 diabetes. There are no approved therapies for NAFLD/NASH, or approved biomarkers to readily assess the disease progression. NASH is diagnosed histologically by assessing inflammation, excess hepatic lipid (steatosis), hepatocyte ballooning, and fibrosis[1,2,3,4]. In clinical trials, these endpoints are assessed prior to the study in a liver sample collected via biopsy, and from a second liver biopsy after a certain treatment period. Thus, the effectiveness of the test agent is assessed via change in the liver pathology in subjects with previously identified/quantified (i.e., biopsy-proven) NASH that are randomized to treatment groups. These same histologic endpoints are often used in rodent models assessing potential NASH therapeutics in preclinical studies, but only in terminal tissue from euthanized mice and thus without an understanding of the degree of efficacy at baseline and an inability to control for differences in baseline disease state between treatment groups.

Although 25% of adults with NAFLD are presumed to have NASH based on elevated serum aminotransferase levels, there are no alternative definitive methods to confirm NASH other than histological evidence[4]. Noninvasive methods, such as radiologic modalities (ultrasound, computed tomography, and magnetic resonance imaging) have been developed to detect hepatic steatosis, but those methods cannot diagnose NASH or determine the stage of fibrosis. Transient elastography offers a promising non-invasive modality for measuring liver stiffness. It has been shown to be accurate in differentiating fibrosis stages. However, the accuracy decreases with higher body mass index (BMI) and fat tissue.[5] This is problematic due to the majority of NASH patients being obese. Beyond the clinical relevance of these approaches, none of these modalities are utilized in preclinical studies.

To overcome this, the research group of Dr. Jonathan Roth were the first to implement a liver biopsy procedure in the Amylin NASH (AMLN) mouse model of NASH[6,7,8]. They described the predictive validity of the biopsy with degree of NASH disease and correlation with metabolic disease. Here we describe in detail the liver biopsy procedure in the AMLN model of NASH and its utility in a therapeutic setting, specifically to assess the efficacy of liraglutide.

Protocol

All in vivo experiments were conducted in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC) of MedImmune, LLC and in agreement with the Guide for the Care and Use of Laboratory Animals, 8th edition (2011). Male C57BL6J mice were placed onto the high-fat, high-fructose, high-cholesterol AMLN diet. Liver biopsies were performed after 26 weeks on diet, 3 weeks prior to the start of the pharmacological intervention.

1. Obtain and Prepare Surgical Materials

1. Autoclave sterile instruments (small pointed-tip scissors, blunt tip forceps, cotton swabs, gauze squares, 7 mm staples/wound clips and stapler).
2. Obtain, sterile suture for closing abdominal wall (5-0 coated absorbable suture with a PC-1 needle is suitable), absorbable gelatin compressed sponge (to be used for hemostatic purposes), sterile drapes and gloves, iodine-based scrub and 70% ethanol swabs/wipes, buprenorphine (0.05-0.1 mg/kg dose per mouse; at least 3 doses per mouse) and surgical eye ointment/lubrication.
3. Prepare vaporizer for anesthesia by filling with isoflurane.
4. Turn on a bead sterilizer, for quick instrument sterilization between animals.
5. Turn on a warming surface or heating pad, to be used for animal recovery.
6. Don appropriate personal protective equipment (PPE) (i.e., mask, gown, hair bonnet, gloves).

2. Pre-surgical Preparations

1. Place the mouse into the induction chamber with 2-3% isoflurane flow to effect.
2. Observe the mouse until the lack of response to toe-pinch confirms the depth of anesthesia is appropriate.
3. Use an eye ointment to prevent dryness of cornea associated with anesthesia.
4. Administer buprenorphine subcutaneously (0.05-0.1 mg/kg).
5. Shave the surgical site immediately caudal of the xiphoid process, using animal clippers.
6. Move the mouse to a heated surface (37 °C) after shaving the surgical site. Place the mouse in such a way so that the dorsal surface is downward, and the abdomen is exposed. Insert the nose of the mouse into the anesthesia nose cone to facilitate delivery of isoflurane at a flow rate of 2-3%.
7. Clean the surgical site with alternating swabs of betadine and 70% ethanol (3 times).
8. Open and set up sterile surgical instruments.
9. Remove gloves and replace with sterile surgical gloves.

3. Surgical Procedure

1. Make a longitudinal incision with scissors (1-2 cm), immediately caudal of the xiphoid process and slightly to the mouse's left side, in the abdominal skin.
2. Blunt dissect the skin away from the abdominal wall using forceps and scissors.
3. Expose the abdominal wall and visualize liver underneath.
4. Using a sterile scalpel blade make an incision (1-2 cm) in the abdominal wall to visualize the left-lateral lobe (LLL) of the liver.
   NOTE: Be consistent throughout the study. Other common lobes of interest are right-medial lobe (RML) and left-medial lobe (LML).
5. Externalize the left lateral lobe through the incision.
6. Option 1: Lightly massage the abdomen with the thumb and index finger to expose a portion of the LLL.
7. Option 2: Place a sterile cotton swab underneath the LLL to gently pull a portion out to be exposed.
8. Place a sterile saline-soaked piece of gauze underneath the LLL to allow a sterile and hydrated surface for the liver to lie on.
9. Cut a wedge-shaped piece of the liver from the LLL by making two cuts with sterile scissors.
   NOTE: The approximate size of the liver being removed is 30-50 mg (<5% of the liver in mice).
10. Cut a piece of gelatin sponge similar in size to the wedge piece of liver. Insert the sponge into the cut portion of the liver using forceps.
11. Hold the sponge in place with forceps and maintain contact with the liver until it adheres to the surface of the liver and hemostasis is achieved.
12. Place the liver biopsy piece into the appropriate tube (i.e. frozen, fixative, etc.).
13. Gently place the liver back into the abdominal cavity, taking care to not disturb the sponge.
14. Suture the abdominal wall with 5-0 coated absorbable suture starting and ending with a full square knot.
15. Close the skin with 7 mm wound clips (or staples).
16. Inspect the wound clips to ensure that there is a complete closure of the incision.
17. Remove the mouse from the nose cone and place into a clean cage on a heated surface.
18. Place instruments into bead sterilizer in between mice for 10-20 s. Make sure all organic material should be removed from the instruments using sterile saline prior to placing in the glass bead sterilizer.

4. Post-operative Care

1. Monitor the mouse until it regains consciousness and can right itself.
2. Record post-operative observations and check the mice daily for dehiscence.
3. Administer a second and third dose of buprenorphine (0.05-0.1 mg/kg) subcutaneously 6-8 h post-surgery and again 24 h post-surgery.
4. Remove wound clips at 7-10 days post-surgery.

Representative Results

For this study, mice were randomized to the treatment groups (n = 12/group) based on pre-study biopsy fibrosis grade and body weight. Mice were administered vehicle (PBS) or liraglutide (5 nmol/kg) for 42 days (vehicle or compound administered 10 mL/kg dose volume, subcutaneously, once daily). On day 42, mice were euthanized (via CO_2 inhalation) in the non-fasted state, and liver excised and processed for histology.

Biopsy and terminal liver pieces were fixed overnight in 10% formalin, then paraffin embedded and processed for hematoxylin and eosin, and Sirius Red, staining via standard protocols[9]. Tissue sections were assessed in a blinded fashion by a pathologist according to the NASH Activity Scoring (NAS) system[1,2]. The NAS reflects the degree of disease for steatosis, lobular inflammation and hepatocyte ballooning (each parameter given a score of 0-3) as previously described[9].

Incorporating a liver biopsy into the design of this study allowed for a baseline (pre-biopsy) fibrosis score. A total of 118 mice were biopsied after 26 weeks of NASH diet and liver fibrosis and steatosis were scored for each biopsy (**Figure 1**). Of these 118 mice, 49 mice were excluded based on low fibrosis (score of 0), high fibrosis (score of 4, which exhibited abnormal pathology), and abnormal weight loss or wound healing. These exclusion criteria allowed for 69 mice left to assign into study groups and look for improvements in NASH phenotype after treatment (**Figure 1**).

Mice were treated with vehicle or the GLP-1R agonist liraglutide (5 nmol/kg) for 6 weeks. In the vehicle-treated group, 1 mouse exhibited worsened fibrosis and 4 mice showed worsened NAS score (**Figure 2A-B**). Liraglutide treatment was associated with an overall improvement of fibrosis (**Figure 2C**), with 17% of the treatment group improving and 83% remaining unchanged. Similarly, liraglutide treatment improved the overall NAS score (**Figure 2D**), with 66% of the group having improved NAS score and 33% remained unchanged.

Additionally, liraglutide treatment improved inflammation, with 75% of the group having a lower inflammation score and 25% remained unchanged (Figure 3B). Vehicle treatment worsened inflammation, with 17% of the group having a higher inflammation score and 83% remained unchanged (**Figure 3A**). Steatosis scoring remained unchanged from baseline to end of the study in both the vehicle- and liraglutide-treated groups (**Figure 3A, B**). Pre- vs. post-biopsy comparisons for individual mice on inflammation and ballooning parameters are also shown (**Figure 3C-F**).

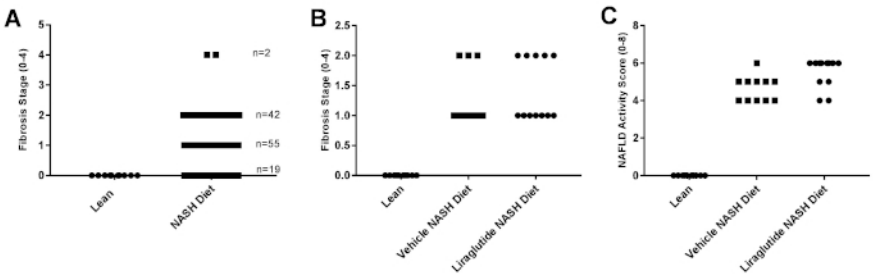

Figure 1: Pre-screening liver biopsy fibrosis and NAS score. (**A**) Representation of all 118 mice that were screened for fibrosis, via liver biopsy. (**B** and **C**) show the baseline fibrosis and NAS score, respectively, after eligible mice were sorted into study groups. Please click here to view a larger version of this figure.

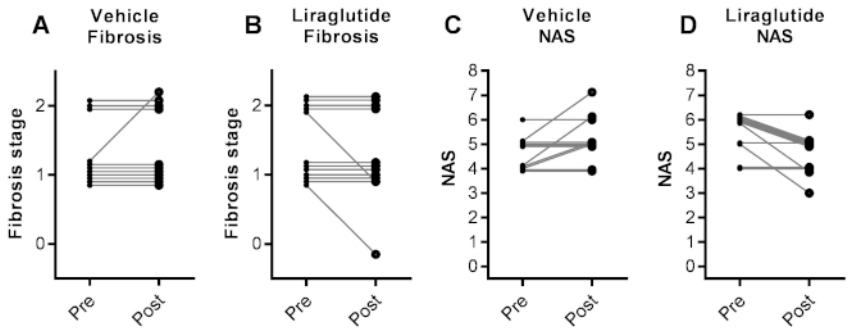

Figure 2: Fibrosis and NAS score, pre-biopsy and post-intervention. Percent responder analysis for the vehicle (**A, C**) or liraglutide-treated (**B, D**) mice, as measured from baseline liver biopsy to end of study liver sample for fibrosis stage (A, B) and NAS (C, D). For each group, the change from the pre-study to post-study biopsy is indicated by a line. The points at each scoring step is slightly shifted to allow visual separation of the animals, this is only for visualization purposes and does not reflect any difference in score. Please click here to view a larger version of this figure.

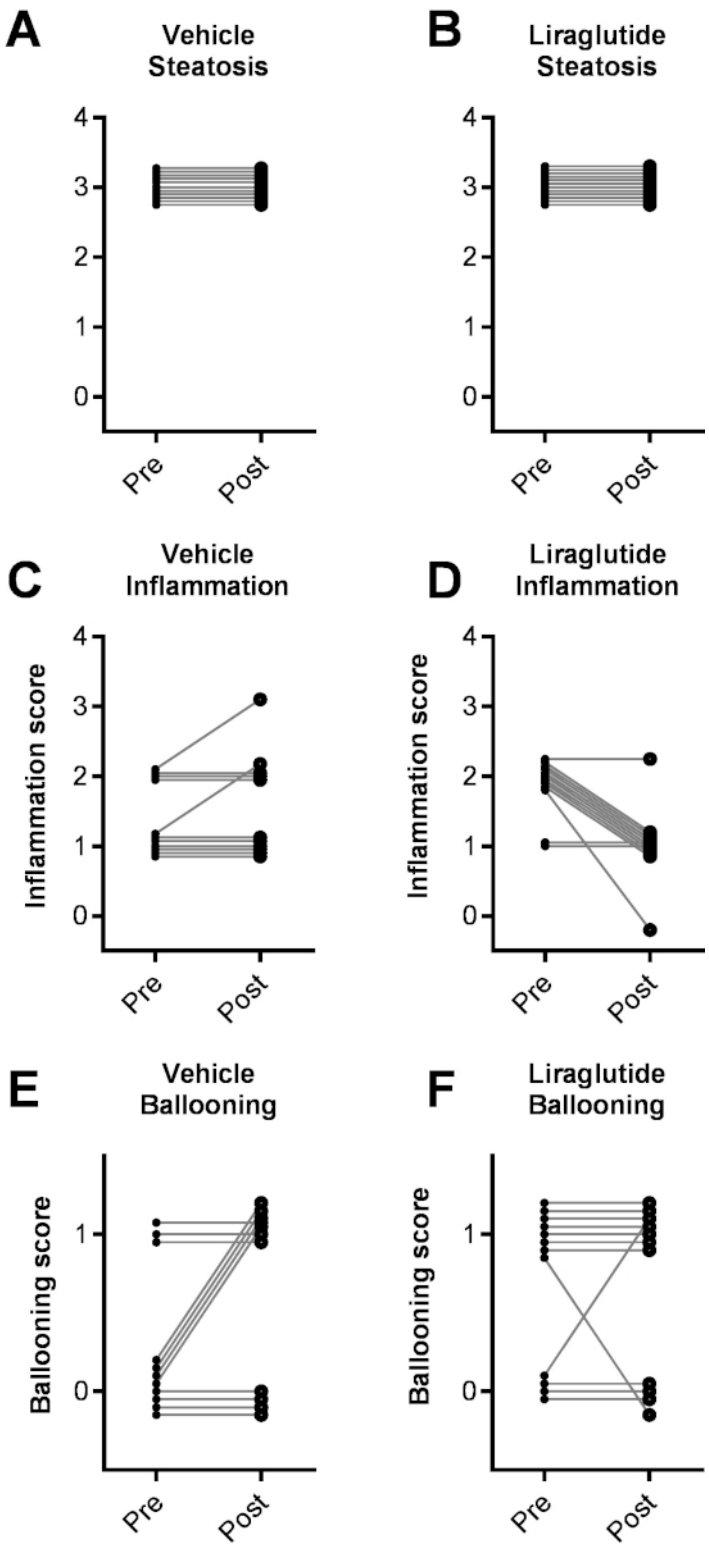

Figure 3: Steatosis, inflammation and ballooning, pre-biopsy and post-intervention. Percent responder analysis for the vehicle (**A, C, E**) or liraglutide-treated (**B, D, F**) mice, as measured from baseline liver biopsy to end of study liver sample for steatosis score (A, B), inflammation (C, D) and ballooning (E, F). For each group, the change from the pre-study to post-study biopsy is indicated by a line. The points at each scoring step is slightly shifted to allow visual separation of the animals, this is only for visualization purposes and does not reflect any difference in score. Please click here to view a larger version of this figure.

Discussion

Here we describe in detail the mouse liver biopsy procedure first described by Clapper et al.[10], and exemplify its utility in a mouse model of NASH, similar to the previous reports[6,9]. The liver biopsy procedure is a valuable tool for assessing NASH phenotype pre- and post-treatment, in a manner similar to that performed in clinical therapeutic trials. The biopsy allows for direct comparison for each subject, i.e. the same mice can be used for both time points. We also demonstrate its translational potential. In a clinical trial in biopsy-proven NASH subjects, liraglutide elicited a 39% response rate (patients with improvement) in NAS, whereas in our study we observed a 66% responder rate for improved NAS. Furthermore, 26% of patients had improved fibrosis score with 9% worsening, versus 14% and 36% in the placebo arm, respectively[11]. In our study, we observed a 17% improvement rate with liraglutide treatment. These comparisons highlight the power of baseline liver biopsy and confirm potential translational understanding, at least for this class of therapeutic.

It is critical to harvest a large enough piece of liver tissue during the biopsy procedure to properly analyze the histological features, but also not too large of a piece of liver to compromise the function of the liver. We observed a zero-mortality rate with this surgery, and the animals appeared fully recovered within 1 day with the resumption of normal eating behavior. Thus, the time between biopsy and initiation of the intervention study is limited not by animal recovery but likely by the time taken for sample processing, analysis, and subsequent randomization.

Utilizing the liver biopsy procedure as a method of confirmation of fibrosis before intervention helped reduce variability in our study, allowing us to exclude mice that did not fully develop some degree of hepatic fibrosis as a result of the AMLN NASH-inducing diet. Furthermore, it enhanced our ability to better understand the impact of possible NASH-improving interventions, as opposed to only having a terminal liver sample and NASH score. Until new non-invasive techniques are further explored and widely accepted, the liver biopsy is the only proven way to determine NASH.

Disclosures

S.O. and C.R. are current employees and/or stockholders of MedImmune/AstraZeneca. JLT was an employee and stockholder of MedImmune/AstraZeneca at the time these experiments were conducted.

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References

1. Brunt, E. M. *et al.* Nonalcoholic fatty liver disease (NAFLD) activity score and the histopathologic diagnosis in NAFLD: distinct clinicopathologic meanings. *Hepatology.* **53** (3), 810-820 (2011).
2. Brunt, E. M. Pathology of nonalcoholic fatty liver disease. *Nature Reviews Gastroenterology and Hepatology.* **7** (4), 195-203 (2010).
3. Kleiner, D. E. *et al.* Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology.* **41** (6), 1313-1321 (2005).
4. Diehl, A. M., & Day, C. Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. *New England Journal of Medicine.* **377** (21), 2063-2072 (2017).
5. Golabi, P. *et al.* Current complications and challenges in nonalcoholic steatohepatitis screening and diagnosis. *Expert Reviews of Gastroenterology and Hepatology.* **10** (1), 63-71 (2016).
6. Roth, N. C. *et al.* Prediction of histologic alcoholic hepatitis based on clinical presentation limits the need for liver biopsy. *Hepatology Communications.* **1** (10), 1070-1084 (2017).
7. Kristiansen, M. N. *et al.* Obese diet-induced mouse models of nonalcoholic steatohepatitis-tracking disease by liver biopsy. *World Journal of Hepatology.* **8** (16), 673-684 (2016).
8. Trevaskis, J. L. *et al.* Glucagon-like peptide-1 receptor agonism improves metabolic, biochemical, and histopathological indices of nonalcoholic steatohepatitis in mice. *American Journal of Physiology Gastrointestinal and Liver Physiology.* **302** (8), G762-772 (2012).
9. Tolbol, K. S. *et al.* Metabolic and hepatic effects of liraglutide, obeticholic acid and elafibranor in diet-induced obese mouse models of biopsy-confirmed nonalcoholic steatohepatitis. *World Journal of Gastroenterology.* **24** (2), 179-194 (2018).
10. Clapper, J. R. *et al.* Diet-induced mouse model of fatty liver disease and nonalcoholic steatohepatitis reflecting clinical disease progression and methods of assessment. *American Journal of Physiology Gastrointestinal and Liver Physiology.* **305** (7), G483-495 (2013).
11. Armstrong, M. J. *et al.* Liraglutide safety and efficacy in patients with non-alcoholic steatohepatitis (LEAN): a multicentre, double-blind, randomised, placebo-controlled phase 2 study. *Lancet.* **387** (10019), 679-690 (2016).