May 28, 2019

Dear Dr. Li-Jun Cui

We appreciate the opportunity to resubmit our manuscript "Prolonged High-Fat-Diet Feeding Promotes NAFLD and Alters Gut Microbiota In Mice" for further consideration for publication in the World Journal of Hepatology.

The revisions to the manuscript are itemized below.

Reviewer #1. Comments for the authors

Reviewer #1: The results of this study suggest that chronic HFD can mimic most of the pathophysiological events observed in NAFLD, such as obesity, steatosis, non-alcoholic stetohepatitis, insulin resistance, steatosis, liver ER stress, and gut dysbiosis. Therefore, chronic HFD is suitable for the establishment of NAFLD model. The paper is well written and is recommended for publication.

Response: We thank this reviewer for the positive comments.

Reviewer #2. Comments for the authors

Reviewer #2: The manuscript by Velazquez et al describes a long term high fat diet model to generate NAFLD and aims to examine the effect of HFD on hepatic histology, metabolic parameters, ER stress, inflammatory pathways and alterations in microbiota composition. The manuscript is clearly written and figures clearly presented. While the authors show significant differences in the observed parameters there are queries (in questions below) in regards to the model and conclusions drawn.

1) Figure 1 graph labels include "Chow" – is this an error, should this read LFD?
    a. If chow is correct then how does composition of the low fat diet compare with a normal chow diet?

Response: This was an error. Figure 1 should read LFD. Our LFD (14% Fat, 54% Carbohydrate, 32% Protein) from Harlan Teklad Rodent no. 8604, commonly known as a chow diet.

    b. Why is chow interchanged with LFD? Likewise what micro- and macronutrient composition differences are there between the LFD and HFD?

Response: We have now used LFD throughout. The kilocalories from protein and carbohydrate are different between the "LFD" and "HFD" diets. For a detailed micro and macronutrient composition of the "LFD" and "HFD" please see the table below. In

general, the main differences between these diets originate from the percentage of kilocalories provided by each macronutrient. The majority of the kilocalories in the "LFD" arises from carbohydrates meanwhile in the "HFD", the majority of kilocalories are derived from fat. We have provided the vendor information and catalog number for the diets so that the reader will have access to the exact diet composition.

	Tekland Rodent Diet	Tekland Rodent Diet	Research Diet	Research Diet
Macronutrients	8604	8604	D12492i	D12492i
	% calories	grams (in 1kg of diet)	% calories	grams
Protein	32	243	20	203
Carbohydrate	54	402	20	247.8
Fat	14	423	60	320
	Tekland Rodent Diet	Tekland Rodent Diet	Research Diet	Research Diet
	8604	8604	D12492i	D12492i
Minerals	%	grams (in 1kg of diet)	%	grams
Calcium	1.4	14	11	5.5
Phosphorus	1.8	18	26	13
Sodium	0.3	3	5.18	2.59
Potassium	1	10	33	16.5
Chloride	0.5	5	5.18	2.59
Magnesium	0.3	3	5.98	2.99
Zinc	0.008	0.08	0.11	0.055
Manganese	0.01	0.1	0.24	0.12
Copper	0.0025	0.025	0.021	0.0105
Iodine	0.0002	0.002	0.001	0.0005
Iron	0.03	0.3	0.42	0.21
Selenium	0.000034	0.00034	0.001	0.0005
	Tekland Rodent Diet	Tekland Rodent Diet	Research Diet	Research Diet
	8604	8604	D12492i	D12492i
Amino acids	%	grams (in 1kg of diet)	% (in 773.85gr in diet)	grams (per 200g Casein)
Aspartic Acid	2.3	23	1.6	12
Glutamic Acid	4.1	41	4.9	37.8
Alanine	1.4	14	0.6	5
Glycine	1.3	13	0.4	3
Threonine	0.9	9	0.9	7.1
Proline	1.6	16	2.3	17.6
Serine	1.6	16	1.3	9.9
Leucine	1.9	19	2.0	15.7
Isoleucine	1	10	1.0	7.5
Valine	1.1	11	1.2	9.2
Phenylalanine	1.1	11	1.1	8.4
Tyrosine	0.9	9	1.2	9.1
Methionine	0.4	4	0.6	5
Cystine	0.4	4	0.5	4.2
Lysine	1.4	14	1.7	13.1
Histidine	0.6	6	0.6	4.5
Arginine	1.5	15	0.8	5.9
Tryptophan	0.3	3	0.3	2.1
	8604	8604	D12492i	D12492i
Vitamins		grams (in 1kg of diet)	%	grams
Vitamin A	12.6 IU/g	0.00000378	0.8	0.008
Vitamin D3	2.4 IU/g	0.00000006	1	0.01
Vitamin E	120 IU/g	0.067	10	0.1
Vitamine K3	40 mg	0.04	0.8	0.008
Vitamin B1	27 mg	0.027	0.6	0.006
Vitamin B2	8 mg	0.008	0.6	0.006
Niacin	63 mg	0.063	3	0.03
Vitamin B6	13 mg	0.013	0.7	0.007
Pantothenic acid	21 mg	0.021	1.6	0.016
Vitamin B12	0.05 mg	0.00005	1	0.01
Biotin	0.38	0.00038	2	0.02
Folate	3	0.003	0.2	0.002
Choline	2530 mg/kg	2.53		2

2) To what extent does differences in carbohydrates rather than fat content affect the liver and gut results observed?

Response: Due to the design of the experiment, we are not able to differentiate if the effects that we are observing are due to high-fat or low-carbohydrate content in the diet. We believe that excess energy consumption resulting from the energy-dense "HFD", is the main factor behind these liver pathologies. Our rational for the experiment was to determine if prolonged-HFD can mimic NAFLD pathophysiology. Studies in low-carbohydrate diets in humans with NAFLD are lacking. However, the benefits of low-carbohydrate diets have been principally associated with the decrease in energy intake [1]. We have now addressed this limitation in the discussion (yellow highlight).

3) What age were the mice at the start of the experiment?

Response: Mice started the experiment at 10 weeks of age. We have now stated the age at which the experiment started in the method section (yellow highlight).

4) A large section of the discussion is simply repeated text (ER stress discussion) – this needs to be corrected. Page 16 is repeated on page 17/18.

Response: We have now eliminated redundant text in the discussion.

5) Can the authors comment on the mild fibrosis and inflammation seen in the young-LFD model? What causes mild hepatic injury in this model in such a small time frame? Are these animals age matched for the start or end of the experiment? Do the authors have measure of liver function such as transaminase levels?

Response: Young-LFD mice showed minimal perisinusoidal fibrosis and inflammation (3 out of 6 mice). Young-LFD mice were 4 months old at the time of euthanasia. Therefore, we believe that the histopathology results of lobular inflammation and mild perisinusoidal fibrosis might be the normal liver histopathological parameters for adult mice as Old-LFD mice and Young-LFD mice have similar lobular inflammation and mild fibrosis scores. Old-LFD and Old-HFD mice were age-matched throughout the experiment. Young-LFD mice were not age-matched to these groups at the start of the experiment. However, Young-LFD mice were euthanized (at 16 weeks of age) on the same dates as Old-LFD and Old-HFD mice. We did not measure the presence of any transaminase in this experiment. In general, Young-LFD mice had a NASH score of 1, which according to Kleiner et al., 2005, is considered not to be diagnostic of steatohepatitis. Comments can be found in methods and discussion (yellow highlight).

6) In Figure 3, what magnification are the inserts and what is being shown in these inserts?

Response: All images in figure 3 were taken at 200X. For manuscript purposes, all images were reduced to fit the paper, except for the inserts (200x magnification). The purpose of the inserts was for the readers to be able to observe at least one hepatocyte and make their own conclusions of the data presented.

7) Can the authors further describe their NASH score, Figure 3J? Is this equivalent to the NAS score as described by Kleiner et al and referred to in reference 21 of the manuscript? Given the significant ballooning, inflammation and steatosis in the old HFD animals wouldn't a higher NAS score be expected? Indeed wouldn't the mild inflammation and steatosis in old LFD animals would generate a NAS score above 0?

Response: We used the Kleiner method to describe NASH. We have now included this reference in the method's section. We agree and thank the reviewer for identifying that we were underestimating the NAS score. We have now corrected Figure 3J and included NAS score interpretation in the methods, results, and discussion (yellow highlight).

8) Can the authors provide further explanation regarding the increased in p-EIF2a in the old LFD group? What other evidence is there that these older animals are in the early stage of chronic ER stress?

Response: We believe that increased phosphorylated- EIF2α reduces chronic ER stress in our Old-LFD mice. The reasoning comes from a study done in NAFLD mice where phosphorylation of EIF2α was inhibited in hepatocytes [2]. In this study, Choi demonstrated that inhibition of EIF2α phosphorylation exacerbates macro-vesicular steatosis, leukocyte infiltration, and fibrosis in NAFLD mice. On the other hand, increased EIF2α phosphorylation protects hepatocytes from ER stress. This has been further explained in the discussion (yellow highlight).

9) What evidence is there for cell death as described as an explanation for the reduced F4/80 expression?

Response: We agree with reviewer; we do not have any evidence of cell death in liver tissue. Therefore, we are eliminating that statement from the discussion.

Reference:

1 Bravata DM, Sanders L, Huang J, Krumholz HM, Olkin I, Gardner CD, Bravata DM. Efficacy and safety of low-carbohydrate diets: a systematic review. *JAMA* 2003; **289**(14): 1837-1850 [PMID: 12684364 DOI: 10.1001/jama.289.14.1837]

2 Choi WG, Han J, Kim JH, Kim MJ, Park JW, Song B, Cha HJ, Choi HS, Chung HT, Lee IK, Park TS, Hatzoglou M, Choi HS, Yoo HJ, Kaufman RJ, Back SH. eIF2alpha phosphorylation is required to prevent hepatocyte death and liver fibrosis in mice challenged with a high fructose diet. *Nutr Metab (Lond)* 2017; **14**: 48 [PMID: 28781602 PMCID: PMC5537942 DOI: 10.1186/s12986-017-0202-6]