Author's response to reviews

Title: High-saturated fat diet delays diethylnitrosamine-induced hepatocellular carcinoma

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A point-by-point reply to reviewers’ comments
Manuscript title: High-Saturate-Fat Diet Delays Development of Diethylnitrosamine-induced Hepatocellular Carcinoma
Manuscript ID: 1388219920112053
Authors: Xiao-Yan Duan, Qin Pan, Shi-Yan Yan, Wen-Jin Ding, Jian-Gao Fan, Liang Qiao
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Comments and suggestions from Reviewer 1 (Steven Dooley)
Comments/Suggestions (Reviewer 1.1): Authors showed that high fat diet can delay HCC development in a rat DEN model. The finding is very interesting and important for HCC research. The current finding is contrary to the finding from Hill-Baskin AE et al, Hum Mol Genet. 2009. Park EJ et al Cell. 2010, Blanche C. Ip et al. The Journal of Nutrition. 2014. However, different models were used. In this paper, authors used a rat DEN HCC model as contrasting with mice DEN HCC models in the other above-mentioned papers. Further, a different genetic background might cause different responses to high fat diet. But, still several major concerns are relevant. These papers should be carefully discussed in the context of the authors’ results.

In the current paper, the authors showed that high fat diet can delay HCC. However, the low fat diet group should also be included, to see whether low fat diet could promote HCC. This will be a valuable information to understand how fat diet can affect HCC development in rats.

Reply: Discussion/comments on discrepancy between our data and the data published in the above-quoted papers have been added to the relevant portion of this revised manuscript. In our study, normal control diet (NCD) mice were fed standard chow consisting of 13.8% calories from fat, 60.5% calories from
carbohydrates, and 25.7% calories from protein, and the high-fat diet (HFD) mice received 10% lard oil, 2% cholesterol and 88% standard chow, with 36.5% calories from fat, 44.6% calories from carbohydrates, and 18.9% calories from protein for 10, 12, and 14 weeks, respectively. As this study mainly focuses on the impact of HFD on the development of HCC, the low-fat diet (<5% of the energy from fat) arm was not included. As a result, all comparisons were made between the NCD and HFD groups. Indeed, a recently published study have revealed that a low-fat intake could lead to the deterioration of energy status in HCC patients, and this was associated with a poor recovery from invasive treatments (Yamada K et al. Nutr J 2013; 12: 79).

Comments/Suggestions (Reviewer 1.2): Authors showed reduced proliferation and increased apoptosis in high-fat diet DEN mice at 10 and 12 weeks. Here, more molecular details should be addressed to find out the mechanism behind the observation. In figure 3, at week 14, apoptosis rate in high fat diet DEN mice is reduced instead of decreased. This should be clarified.

Reply: These comments are very good ideas to improve the quality of the manuscript. We have begun to do some molecular details behind the observation, however, we have not received good results. It is true that apoptosis rate in HFD DEN mice is significantly reduced. We have explained the dynamic changes of the apoptosis in the revised manuscript.

Comments/Suggestions (Reviewer 1.3): In figure 1, pictures of the whole liver should be provided to show the difference.

Reply: The pictures of the whole livers from NCD and HFD with DEN groups at the end of 10, 12, and 14 weeks were shown in supplement figure at the end of the reply, and the representative photos from each group have been added to the revised Figure 2.

Comments/Suggestions (Reviewer 1.4): In figure 2a, data from all individual mice should be shown as immunoblot for PCNA.

Reply: Data from all individual mice have been shown as immunoblot for PCNA, see revised Figure 3a.

Comments/Suggestions (Reviewer 1.5): In figure 2a and 3, immunohistochemistry staining of PCNA and Caspase 3 should be shown in order to find out the cell type and expression area in the animals. Proliferation markers such as ki67 or Tunel staining should also be provided.

Reply: It is a good suggestion, however, the immunohistochemistry staining of PCNA and Caspase 3, and proliferation markers such as ki67 and Tunel staining have not done in this study at the end of 10, 12, 14 weeks. We have explained such limitation in the revised manuscript.

Comments/Suggestions (Reviewer 1.6): In tables 2 and 3, representative photos of fibrosis and differentiation should be included. Furthermore, inflammation should also be analyzed. Park EJ et al Cell. 2010 showed that enhanced production of IL-6 and TNF is import for high fat diet induced HCC in mice. Here, the authors should find out whether the situation is different in rats and include mRNA/ELISA data for IL6 and TNFa.
Reply: Representative photos of liver fibrosis as assessed by VG stain and
differentiation degree of heptocellular carcinoma have been added to the Figure
1. Furthermore, chronic hepatitis activity index (HAI) proposed by Knodell was
adopted to calculate the liver inflammatory activity score in the revised
manuscript. Therefore, we could analyze the impact of inflammation on the
initiation of HCC in DEN mice fed with or without HFD, our findings are different
with the study by Park EJ et al, as HAI in HFD group is significantly less than that
in NCD group at the end of 10, 12 weeks, even at the end of 14 weeks, HAI in
HFD group is slightly less than that in DEN group. More details see the below
table.

<table>
<thead>
<tr>
<th>Time</th>
<th>Group</th>
<th>N</th>
<th>HAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DEN+CD</td>
<td>7</td>
<td>12.1±2.2</td>
</tr>
<tr>
<td></td>
<td>DEN+HFD</td>
<td>7</td>
<td>10.7±2.9</td>
</tr>
<tr>
<td></td>
<td>12W DEN+CD</td>
<td>7</td>
<td>14.9±4.5</td>
</tr>
<tr>
<td></td>
<td>DEN+HFD</td>
<td>7</td>
<td>11.0±1.2#</td>
</tr>
<tr>
<td></td>
<td>14W DEN+CD</td>
<td>9</td>
<td>15.1±3.1</td>
</tr>
<tr>
<td></td>
<td>DEN+HFD</td>
<td>10</td>
<td>14.0±2.3</td>
</tr>
</tbody>
</table>

# P#0.05

Comments/Suggestions (Reviewer 1.7): How many animals were used per time
point in each of the analyses? This should always be stated in the figures.

Reply: This point has been clarified in the revised manuscript.

Minor Essential Revisions

Comments/Suggestions (Reviewer 1.8): …..missing labels on figures, the wrong
use of a term, spelling mistakes. In figure 2a, molecular weight of PCNA and
actin should be mentioned on the picture.

Reply: Necessary corrections/revisions have been done.

Comments/Suggestions (Reviewer 1.9): In figure 2b, 2c and 3, dot graph would
be better than column to show individual animals.

Reply: Revised accordingly.

Comments/Suggestions (Reviewer 1.10): Reference 19 and 33 is exactly the
same.

Reply: Redundant reference has been removed.

Comments and suggestions from Reviewer 2 (Hirofumi Uto)

Comments/Suggestions (Reviewer 2.1): The effect of HFD in this study may
depend on total calorie intake rather than fat intake. In fact, HFD restored
malnutrition in the DEN-treated rats as stated by authors. Authors should justify
the total calorie intake in the HFD+DEN group and NCD+DEN group.

Reply: We can not exclude that the observed effect of the HFD was exclusively
due to high fat intake, as the rats in the DEN+HFD group had more daily food
intake than those in the DEN + NCD group (Table 1). However, as each diet
contains the same amount of total calorie, we believe the observed effect could be mostly attributed to the difference in the dietary fat between these 2 diets, and partly related to the total calorie intake.

Comments/Suggestions (Reviewer 2.2): Rahman et al concluded that the effect of dietary fat during the initiation phase of AOM-induced hepatocarcinogenesis depends on the type of fat and its fatty acid composition (Ref 18). Sugie et al also showed that the density and the unit area of AOM-induced enzyme altered foci in the liver were significantly lower in the high fish oil group than in the 5% corn oil group and the low fish oil group (Nutr Cancer. 1995;24:187-95.). In addition, Rahman et al reported that fish oil rich in polyunsaturated omega-3 fatty acids could inhibit DEN-induced hepatocarcinogenesis in rats (Jpn J Cancer Res. 1999;90:31-9.). If the effect of HFD in this study depends on fat rather than total calorie intake, authors should clarify whether high-saturate-fat or high-unsaturated-fat is better to inhibit DEN-induced hepatocarcinogenesis.

Reply: In the original experimental design, we aimed to study the effect of saturated fatty acids on DEN-induced hepatocarcinogenesis. The authors appreciate the suggestion of this reviewer that the effect of unsaturated fatty acids should also be investigated. This theme would be our future research topic.

Comments/Suggestions (Reviewer 2.3): Authors showed the anti-proliferative and pro-apoptotic effect of HFD in rat liver. Are these effects observed in both tumor cells and non-tumor cells (normal hepatocytes)?

Reply: Yes, the presented data were derived from tumor cells and non-tumor cells.

Comments/Suggestions (Reviewer 2.4): There was an increase in the hepatic level of caspase-3 in the HFD+DEN group compared to the NCD+DEN group at weeks 10 and 12, and authors concluded that anti-apoptotic effect may be associated with the attenuation of hepatocarcinogenesis. However, the hepatic level of caspase-3 in the HFD+DEN group was significantly lower than that of the NCD+DEN group at weeks 14.

Reply: Please refer to the Reply to Comments/Suggestions (Reviewer 1.5).

Comments/Suggestions (Reviewer 2.5): Authors stated that there was a more significant reduction in the PCNA expression in HFD+DEN group at weeks 12 and 14 compared to rats in the NCD+DEN group (page 7, line 23-). However, the PCNA expression (hepatic content of PCNA) in HFD+DEN group at weeks 14 was relatively higher than those in the NCD+DEN group (Fig 2 C).

Reply: Necessary rewording has been done in the revised manuscript. Please also refer to the Reply to Comments/Suggestions (Reviewer 1.5).

Comments/Suggestions (Reviewer 2.6): Authors stated that HFD appeared to attenuate the occurrence of HCC and malignant differentiation in rat HCC model induced by DEN (Page 9, 12-). Three steps of progression of hepatocarcinogenesis have been proposed: initiation, promotion, and progression. Authors should discuss this point more in detail.

Reply: Appropriate discussion to this stepwise hepatocarcinogenesis model has been added to the relevant portion of the revised manuscript.
Comments and suggestions from Reviewer 3 (Alexander Wree)

Comments/Suggestions (Reviewer 3.1): The abstract provided does not give any background and introduction to the topic. The sentence given with the heading background describes the aim of the study. Moreover, authors introduce the abbreviation DNE for diethylnitrosamine.

Reply: The abstract has been revised and the wrongly spelled words have been corrected.

Comments/Suggestions (Reviewer 3.2): Based on which rationale did the authors choose the time points 10, 12, and 14 weeks? DEN has been used for a broad range of time points in rats anywhere from 8 weeks to 16 months (e.g. Taya et al 2014, Carthew et al 1997, Takahashi et al. 1984).

Reply: The impact of HFD on DEN-induced hepatocarcinogenesis was dynamically studied. We chose week 10 as the starting point and week 14 as the endpoint of analysis. This is because all rats developed HCC by week 14, and the effect of HFD on liver pathology becomes evident during this time period. In addition, we have done the experiment of 16 weeks of DEN with and without HFD before chosen the time points, maintaining the experimental rats beyond week 16 was not ethically permitted because the general condition of all rats deteriorates thereafter.

Comments/Suggestions (Reviewer 3.3): Authors extensively describe the liver histology in mice fed with NCD and HFD with or without DEN treatment. Therefore, macroscopic images of livers should be presented in Figure 1 along with microphotographs in high, as well as in low magnifications.

Reply: Please refer to the Reply to Comments/Suggestions (Reviewer 1.3).

Comments/Suggestions (Reviewer 3.4): Did the authors perform any specific staining to assess liver fibrosis, e.g. Sirius Red Staining or Masson's trichrome? If so, this should be presented to the readership.

Reply: Data from VG staining for liver fibrosis were added. Please refer to Reply to Comments/Suggestions (Reviewer 1.6).

Comments/Suggestions (Reviewer 3.5): Authors state in the Method section that a commercial enzyme-linked immunosorbent assay (ELISA) kit was used to quantify the active form of Caspase 3. However, in the Results, as well as the Figures, they report the total content of Caspase 3. Authors emphasize the resistance to apoptotic cell death as an important contributor to the documented phenotype. How do authors explain that the hepatic content of Caspase 3 is increased in measurements at week 10 and 12, while decreased at 14 weeks in the HFD+DEN group when compared to the NCD+DEN group?

Reply: This aspect has been discussed in the Discussion of the revised manuscript. Please also refer to the Reply to Comments/Suggestions (Reviewer 1.5).

Comments/Suggestions (Reviewer 3.6): In previous studies, DEN has been administered via gavage, peritoneal injection, or tail vein injection. The main effect reported in the presented study is addressed to a dietary intervention. How
can the authors exclude that the diet itself does not interfere with the DEN? Is the beneficial effect of HFD still present when DEN is administered via a non-oral path?

Reply: In this study, DEN was administered by gavage and the impact of HFD on DEN-induced hepatocarcinogenesis was examined. The impact of other dietary components on the carcinogenic effect of DEN as well as the effect of different routes of administration on the combinatorial effect of HFD and DEN are beyond the scope of this study. These ideas may form good rationales for future studies.

Comments/Suggestions (Reviewer 3.7): Page 5, line 1: “Metavir Score system”. An appropriate reference should be added.


Comments/Suggestions (Reviewer 3.8): Page 6, line 10: “as shown in Table 4”, do the authors mean Table 1 or do they refer to additional table.

Reply: This confusion has been clarified in the revised manuscript.

Comments/Suggestions (Reviewer 3.9): Figures 2 and 3: Number of mice per group analysed in panel B and C should be given.

Reply: The number of mice per group analysed in Figures 2 and 3 have been given in the revised manuscript.