Response to Reviewers' Comments

Manuscript Title: Three-dimensional perfused human in vitro model of non-alcoholic fatty liver disease

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General comments

Authors showed develop a human in vitro model of non-alcoholic fatty liver disease by using primary hepatocytes cultured in a three-dimensional perfused platform. In this model, hepatocytes cultured in fat medium were found to accumulate fat more than lean cells although ALT and AST did not change. Furthermore, inflammatory and fibrotic genes associated with NAFLD were upregulated in hepatocyte cultured in fat medium. Finally, authors concluded that the 3D in vitro NAFLD model recapitulates many features of clinical NAFLD and is an ideal tool for analyzing the efficacy of anti-steatotic compounds. This original paper is interesting, and manuscript is well written.

> **Response**: The authors would like to thank the reviewer for their comments and have made amendments to the manuscript and highlighted the changes in yellow throughout the paper.

Reviewer major comments:

1. *Authors demonstrated that fat accumulation and the levels of free fatty acids increased in hepatocyte cultured in fat medium. However, the cytotoxic effect did not change in this system. Authors should discuss about these points. Why do free fatty acids increase in these conditions? From where do free fatty acids increase?*

> **Response**: The hepatocytes cultured in the model did not show any signs of cytotoxicity when cultured in fat medium. However, it is known that when lipids over accumulate in non-adipose tissues, they can enter non-oxidative deleterious pathways, leading to cell injury and death. Previous studies have shown that fat loading of hepatocytes cultured *in vitro* can cause cytotoxicity, particularly when high concentrations of saturated fatty acids are present (Gomez-Lechon MJ, et al. 2007. *Chem Biol Interact*; 165(2): 106-116). The concentration of free fatty acid (600 μM) and ratio of saturated to unsaturated free fatty acids (1:2) used in our model was specially chosen as these had previously been previously shown to not cause cytotoxicity, whilst inducing significant levels of intracellular triglyceride accumulation in primary hepatocyte cultures (Chavez-Tapia NC, et al, 2012; *BMC Gastroenterol*; 12: 20) (Cui W, et al. 2010; *Am J Transl Res*; 2(1): 95-104) (Gomez-Lechon MJ,

et al. 2007. *Chem Biol Interact*; 165(2): 106-116). This important discussion point has now been included in the manuscript.

Figure 2C demonstrates that hepatocytes continue to consume free fatty acid over the 14-day culture period. Fatty acid consumption was determined by measuring changes in the concentration of free fatty acid in the cell culture medium. The loss of free fatty acid from the media is considered to be the amount of free fatty acid consumed by the cells. The loss of free fatty acid from the media was calculated by measuring concentrations in the input media (600µM) and medium removed from the culture plates at each media change (typically 100-500µM). As is standard practice the cell culture medium in each bioreactor was changed completely every 2-3 days, providing the cells with fresh cell culture media and therefore more free fatty acids to consume. This process is explained in the "Hepatocyte culture" section and "Free fatty acid consumption" sections of the Material and Methods, but has now also been further explain in the Results section.

2. *Several inflammatory genes associated with NAFLD were increased in this system. However, authors should discuss regarding the other inflammatory genes including IL-1β, IL-6 did not change in this system. Why does only IL-8 and MIF increase in this system?*

Response: Figure 4 demonstrates the changes that occur to the adipokines that are expressed by the hepatocytes cultured in the model and released into the culture medium. We have highlighted the adipokines that are altered by the accumulation of triglycerides in the cells, but as the reviewer rightly points out a number of other inflammatory cytokines do not change in the systems, including IL-1β and IL-6. This observation is in part due to the model described being a monoculture of hepatocytes and many inflammatory signalling molecules present in the liver, originate from non-parenchymal cells. For example, IL-6 is well known to be produced by non-parenchymal cells in the liver which is then detected by hepatocytes via the IL-6 receptor and the glycoprotein Gp130 (Schmidt-Arras D, Rose-John S, 2016; *J Hepatol;* 64: 1403-1415). The adipokines observed to change in the model including IL-8, fibrinogen, and MIF are known to be expressed at detectable levels by hepatocytes and are all associated with a NAFLD phenotype (Joshi-Barve S, et al 2007; *Hepatology*; 46(3): 823-830) (Braunersreuther V, et al. 2012; *World J Gastroenterol*; 18(8): 727-735) (Morrison MC, Kleemann R, 2015; *Front Immunol*; 6(308) 1-13). Additionally, for most inflammatory signals to be released in the liver, a more potent stimulus than simple triglyceride accumulation is required. As a result, simple steatosis is described as a benign disease state and only when other signals (e.g. from the gut) promote further inflammation in the liver that the disease state progresses.

3. *What does the increment of MIF mean?*

Response: In the model described fat accumulation of hepatocytes was observed to increase the production of macrophage migration inhibitory factor (MIF). MIF is a cytokine that is expressed both by immune and non-immune cells and is known for its pro-inflammatory effects and its ability to negatively regulate the immunosuppressive actions of glucocorticoids. Increased MIF expression is associated with NAFLD development *in vivo*,

with liver biopsies of NAFLD patients showing higher expression of MIF compared to healthy controls. Animal studies have also shown an association between high fat diets, insulin resistance and the plasma level of MIF (Morrison MC, Kleemann R, 2015; *Front Immunol*; 6(308) 1-13). The reviewer correctly points out that this discussion of MIF was not present in the manuscript, but it has now been included.

4. *Metformin or PGZ showed anti-steatotic effect in this system. Does this effect correlate with the suppression of inflammatory and fibrosis marker? Authors can check the effect of metformin and PGZ on inflammation and fibrosis in this system.*

Response: Treatment with both Metformin and PGZ had anti-steatotic effects in the system, but the authors did not observe a correlation with a suppression in inflammation and fibrosis markers. The authors have observed that fat loaded samples with and without PGZ treatment had equivalent levels of IL-8 and fibrinogen (data not shown). We have noted this observation in the results section of the manuscript.

5. *Please show the reason why authors select the concentration of metformin (100 uM) or PGZ (1-10 uM). What is the action site in PGZ?*

Response: The concentrations of both metformin and PGZ were chosen from searching the literature for similar types of study (Qin et al 2007, *Arterioscler Thromb Vasc Biol*; 27(11): 2428-2434), (Sakamoto et al 2000 *Biochem Biophys Res Commun*; 278(3): 704-711), (He and Wondisford, 2015, *Cell Metabolism*; 21: 159-162). We were also keen to use physiologically relevant concentrations of both drugs, which were representative of therapeutic doses achievable *in vivo*. In particular, many previous *in vitro* studies have used Metformin at supra-pharmacological concentrations 10-100 times higher than those used in patients (He and Wondisford, 2015, *Cell Metabolism*; 21: 159-162). We have included this point in the results section of the manuscript.

The action site of PGZ is the activation of PPARγ, which alters the lipid metabolism and lipoprotein lipase expression in hepatocytes. We have highlighted this in the discussion.

Reviewer Minor point:

1. *Authors should show the composition of lean media.*

Response: The composition of lean and fat media is included in the Hepatocyte culture section of the Material and Methods:

"cells were cultured in lean or fat WEM, both of which contained physiologically relevant quantities of insulin (2 nM) and glucose (5.5 mM), as well as standard WEM supplements (0.5% Pen/Strep, 2 mM GlutaMAX, 15 mM HEPES, 6.25 ng/mL sodium selenite, 6.25 µg/mL transferrin, 1.25 mg/mL BSA, 5.35 µg/mL linoleic acid, 100 nM dexamethasone). The fat media was further supplemented with 600 µM free fatty acids, containing a 2:1 mix of oleate acid and palmitate acid, conjugated to BSA."