Journal of Visualized Experiments

A model of experimental steatosis in vitro: hepatocyte cell culture in lipid overload-conditioned medium

--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62543R2
Full Title:	A model of experimental steatosis in vitro: hepatocyte cell culture in lipid overload-conditioned medium
Corresponding Author:	Carolina Guzman Hospital General de México "Dr. Eduardo Liceaga" Mexico City, Cuauhtémoc MEXICO
Corresponding Author's Institution:	Hospital General de México "Dr. Eduardo Liceaga"
Corresponding Author E-Mail:	carova@prodigy.net.mx
Order of Authors:	Adriana Campos-Espinosa
	Carolina Guzman
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please specify the section of the submitted manuscript.	Medicine
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Alcaldia Cuauhtemoc, Mexico City, Mexico
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

A Model of Experimental Steatosis *In Vitro*: Hepatocyte Cell Culture in Lipid Overload-Conditioned Medium

AUTHORS AND AFFILIATIONS:

Adriana Campos-Espinosa, Carolina Guzmán

Laboratorio de Hígado, Páncreas y Motilidad, Unidad de Medicina Experimental, Facultad de Medicina-UNAM, Hospital General de México "Dr. Eduardo Liceaga", Mexico City, Mexico.

Email addresses of co-authors:
Adriana Campos-Espinosa (adrian_cea@comunidad.unam.mx)
Carolina Guzmán (carolina.guzman@quimica.unam.mx)

Corresponding authors:
Carolina Guzmán (carolina.guzman@quimica.unam.mx)

SUMMARY:

This protocol is intended to be a tool to study steatosis and the molecular, biochemical, cellular changes produced by the over exposure of hepatocytes to lipids *in vitro*.

ABSTRACT:

Metabolic dysfunction-associated fatty liver disease (MAFLD), previously known as non-alcoholic fatty liver disease (NAFLD), is the most prevalent liver disease worldwide due to its relationship with obesity, diabetes type 2, and dyslipidemia. Hepatic steatosis, the accumulation of lipid droplets in the liver parenchyma, is a key feature of the disease preceding the inflammation observed in steatohepatitis, fibrosis, and end-stage liver disease. Lipid accumulation in hepatocytes might interfere with proper metabolism of xenobiotics and endogenous molecules, as well as to induce cellular processes leading to the advance of the disease. Although the experimental study of steatosis can be performed *in vivo*, *in vitro* approaches to the study of steatosis are complementary tools with different advantages. Hepatocyte culture in lipid overload-conditioned medium is an excellent reproducible option for the study of hepatic steatosis allowing the identification of cellular processes related to lipid accumulation, such as oxidative and reticular stresses, autophagia, proliferation, cell death, etcetera, as well as other testing including drug effectiveness, and toxicological testing, among many other possible applications. Here, it was aimed to describe the methodology of hepatocyte cell culture in lipid overload-conditioned medium. HepG2 cells were cultured in RMPI 1640 medium conditioned with sodium palmitate and sodium oleate. Importantly, the ratio of these two lipids is crucial to favor lipid droplet accumulation, while maintaining cell proliferation and a moderate mortality rate, as occurs in the liver during the disease. The methodology, from the preparation of the lipid solution stocks, mixture, addition to the medium, and hepatocyte culture is shown. With this approach, it is possible to identify lipid droplets in the hepatocytes that are readily observable by Oil-red O staining, as well as curves of proliferation/mortality rates.

INTRODUCTION:

Fatty liver associated with metabolic dysfunction is highly prevalent worldwide[1,2]; it is estimated that up to 25% of the population is affected[3]. This disease previously known as non-alcoholic fatty liver disease (NAFLD), has updated its nomenclature to metabolic dysfunction associated fatty liver disease (MAFLD) to accurately reflect the pathogenesis related with obesity, insulin resistance, diabetes type 2, and dyslipidemia, as well as the possible managements of the disease[3,4].

Regardless of the name, the disease includes a wide spectrum of histopathological changes characterized by abnormally high accumulation of lipids in the liver (>5% of fat in the hepatocytes[5]) and might progress through the lipid accumulation typically found in simple steatosis to steatohepatitis, which in turn might lead to the development of fibrosis, cirrhosis, hepatocellular carcinoma, and liver failure[5–8]. Due to its increasing prevalence, MAFLD is expected to become the first indication of liver transplantation and the leading cause of hepatocellular carcinoma[9].

Although it has been considered as a benign or mild form of fatty liver disease, hepatic steatosis is in fact the metabolic key in MAFLD[10]. Different metabolic pathways are affected by lipid accumulation in the liver, including but not limited to lipid synthesis, exportation, and metabolism[10]. Insulin resistance, oxidative stress, reticular stress, and cellular dysfunction are strongly associated to hepatic lipotoxicity[11,12]. On the other hand, fatty hepatocytes are the target of reactive oxygen species, rendering metabolites as lipid peroxides, protein carbonyls, and adducts of nucleic acids[13]. At the cellular level, fatty hepatocytes might undergo mitochondrial damage[14], cellular senescence[15], apoptosis[16], pyroptosis[12], and autophagia[17], among other events.

Hepatocytes are highly responsible for metabolism, detoxification, and synthesis of a wide range of molecules. Many of these functions might be compromised by the lipid accumulation observed in steatosis. Therefore, it is of great importance to have reproducible tools that allow an accurate evaluation of steatosis. In this sense, *in vitro* models are readily applicable and highly reproducible. Steatosis *in vitro* has been used with different goals[16,18,19]. The HepG2 cells are widely used as hepatocyte cell line. It has advantages such as being easy to culture and well characterized. Perhaps, the only disadvantage of HepG2 cells is the fact that it is a carcinogenic cell line, so this must be considered when analyzing the outcomes. Here, the application of a mixture of fatty acids widely used in cell culture: palmitic acid (PA) and oleic acid (OA) is shown. Both PA and OA offer different outcomes in culture[20]. PA (C 16:0) is the most common saturated fatty acid obtained from the diet[16]. PA is considered as a biomarker of *de-novo lipogenesis*, a crucial step in the development of NAFLD[21]. PA is shown to be highly toxic[22]; therefore, it might not be recommended to induce steatosis *in vitro*. OA (C 18:1) is a monounsaturated fatty acid. In contrast to PA, OA has been suggested to possess anti-inflammatory and anti-oxidant properties, being able to counteract PA[12]. Both PA and OA are the main fatty acids present in the triglycerides, regardless of the condition of health or disease[16]. **Table 1** provides examples of the hepatocyte culture with PA, OA, and their mixture,

as well as the outcomes reported[12,23–27]. Other fatty acids have also been used in hepatocyte culture, including stearic acid (C 18:0)[28–30], linoleic acid (C 18:1)[28,30,31] and its conjugates (CLA)[28,32], palmitoleic acid (C 16:1)[29]. However, their use is least frequently reported in the literature, perhaps because their hepatic abundance is lower than PA and OA[16].

In conjunction, both fatty acids resemble steatosis *in vitro*, providing proliferating cells, with increased cell death and lower viability compared with control conditions. It is worth mentioning that the respective salts of these fatty acids are available and can be used as well. One of the main problems when assessing lipid overload in hepatocyte cell culture is given in the differentiation between toxicological models and a model that best represent steatosis. Many models can be accounted in the first case. In fact, the use of PA alone might be considered among them, and the high mortality is the most evident outcome[12,16,23–27]. The use of high doses even in the case of OA can also be considered as a toxicologic model. The protocol shown here is in higher accordance with steatosis development since it shows low mortality compared with that observed in other models and allows it to be followed during several days with progressive lipid accumulation as it occurs in NAFLD. The possibility to assess mild and severe steatosis through experimental conditions is considered another advantage.

[Place **Table 1** here]

Finally, this model is applicable not only to the study of steatosis and fatty liver, but also to the hepatic metabolic, synthetic, and detoxification pathways in the context of steatosis. Also, *in vitro* induced steatosis might provide evidence for the identification of potential markers of the disease as well as therapeutic targets.

PROTOCOL:

1. Standard and conditioned medium preparation

1.1. To prepare standard RPMI 1640 supplement, mix RPMI 1640 culture medium with 10% (v/v) of fetal bovine serum (FBS, previously heat inactivated) and 1% (v/v) of Penicillin-Streptomycin solution. Store the supplement at 4 °C.

1.2. To prepare palmitate stock solution, prepare a 50 mM solution of palmitate in the standard RPMI 1640 previously supplemented with 1% of bovine serum albumin (lipid free). A volume of 5-10 mL of this stock will be sufficient. Sterilize the stock solution by using 0.22 µm filters. Store at 4 °C protected from light for up to 1 month.

1.3. To prepare oleate stock solution, prepare a 50 mM solution of oleate in the standard RPMI 1640 previously supplemented with 1% of bovine serum albumin (lipid free). A volume of 10 mL will be sufficient. Sterilize the stock solution by using 0.22 µm filters. Store at -20 °C protected from light for up to 1 month.

1.4. To prepare steatogenic medium from the previously prepared stocks, prepare a 1-part palmitate: 2-part oleate steatogenic medium at two possible levels: mild and severe steatosis.

1.4.1. Mild Steatosis*:* Prepare 100 mL of a 1-part palmitate: 2-part oleate (50 µM) mix in standard RPMI 1640. Sterilize by using 0.22 µm filters. Store at 4 °C for up to 1 week.

1.4.2. Severe Steatosis*:* Prepare 100 mL of a 1-part palmitate: 2-part oleate (500 µM) mix in standard RPMI 1640. Sterilize by using 0.22 µm filters. Store at 4 °C for up to 1 week.

1.4.3. Alternative preparation for the stock solutions.

1.4.3.1. Prepare both stock solutions using the respective fatty acids by using free lipid albumin as indicated above.

1.4.3.2. When lacking free lipid albumin, use palmitate and oleate salts.

1.4.3.2.1. Dissolve either palmitate or oleate in 2 mL of absolute ethanol and then mix in the final volume of standard RPMI 1640 (5–10 mL). Dissolve oleate directly by stirring in standard RPMI 1640 culture medium.

1.4.3.2.2. Allow evaporation of ethanol by incubating in a water bath at 70 °C; mix thoroughly.

1.4.3.3. In every case, sterilize both stock solutions using 0.22 µm filters. Store palmitate stock solution at 4 °C and oleate stock solution at - 20 °C. Protect both solutions from light. These solutions are stable for 1 month.

2. Pre-culture

2.1. Seed 100,000 HepG2 cells per well in a 24-well plate. Add 1 mL of standard RPMI 1640.

2.2. Pre-incubate at 37 °C and 5% CO_2 for 24 h, allowing cell attachment and confluency of approximately 75%.

3. Steatogenic culture

3.1. After pre-culture, discard the standard RPMI 1640 medium and add the steatogenic medium accordingly.

3.2. Discard the supernatant and add fresh steatogenic medium every 24 h.

4. Viability and mortality assessment

4.1. Seed 100,000 HepG2 cells per well in a 24-well plate. Add 1 mL of standard RPMI 1640.

4.2. Pre-incubate for 24 h at 37 °C and 5% CO_2.

4.3. Change standard RPMI 1640 medium for the steatogenic medium.

4.4. Incubate for 24 h, 2 days, 3 days, and 4 days refreshing the steatogenic medium every 24 h.

4.5. After the appropriate time, discard the supernatant.

4.6. Detach cells from the well by adding 500 µL of 0.05% of Trypsin-EDTA. Incubate for 5 min at 37 °C and 5% CO_2.

4.7. Collect the resuspended cells in a microtube.

4.8. Centrifuge at 300 x *g* and discard the supernatant.

4.9. Add 200 µL of standard RPMI 1640 and resuspend the cells.

4.10. Add 15 µL of 0.4% solution of Trypan blue in a fresh microtube. Mix with 15 µL of the previous cell suspension.

4.11. Count the stained and non-stained cells in a hemocytometer.

4.12. Calculate the viability and mortality rates accordingly.

$$\% \text{ Viability} = \left(\frac{Number\ of\ Live\ cells}{Number\ of Live\ cells + Number\ of\ Dead\ cells}\right) \text{x } 100$$

$$\% \text{ Mortality} = \left(\frac{Number\ of\ Dead\ cells}{Number\ of\ Live\ cells + Number\ of\ Dead\ cells}\right) \text{x } 100$$

5. Lipid staining with Oil-Red O

5.1. Put a cell culture coverslip in every well in a 24-well plate.

5.2. Seed 100,000 HepG2 cells per well. Add 1 mL of standard RPMI 1640.

5.3. Pre-incubate at 37 °C and 5% CO_2 for 24 h.

5.4. Change standard RPMI 1640 medium for the steatogenic medium.

5.5. Incubate for 24 h, 2 days, 3 days, and 4 days, refreshing the steatogenic medium every 24 h.

5.6. After the appropriate time, discard the supernatant.

5.7. Wash with 1 mL of phosphate buffered saline (PBS). Discard the supernatant.

5.8. Fix with 1 mL of 4% paraformaldehyde in PBS.

5.9. Incubate for 1 h at room temperature.

5.10. Discard the excess of paraformaldehyde.

5.11. Rinse the cells with 1 mL of distilled water.

5.12. Add 1 mL of 70% isopropanol and incubate for 5 min.

5.13. Discard the excess of isopropanol. A PBS wash is not needed at this point.

5.14. Add 1 mL of Oil-red O solution and incubate for 30 min.

5.15. Discard the excess of the Oil-red O solution.

5.16. Rinse with 1 mL of distilled water.

5.17. Add 500 µL of hematoxylin solution. Incubate for 3 min.

5.18. Discard the excess of hematoxylin solution.

5.19. Rinse with 1 mL of distilled water.

5.20. Observe under the microscope at a magnification of 400x (Objective 40x, Ocular 10x).

6. Morphometric assessment of lipid contents

6.1. Randomly select and capture photographs of 10 optical fields from the complete area of the well. Repeat for every well.

6.2. Assess the percentage of red stained area using the **Color Threshold** tool in the ImageJ software according to Ferreira and Rasband[33].

6.3. Compare the stained area with the complete area of the optical field using the **Analyze Particles** tool in the ImageJ software according to Ferreira and Rasband[33].

6.4. Calculate the average percentage of every well.

REPRESENTATIVE RESULTS:

Hepatocytes cultured in the steatogenic medium display growth all over the surface of the well; however, fatty hepatocytes show lower growth rate compared with cells cultured in control medium. The proposed ratio and concentration of OA and PA, guarantee cell survival during culture. Seeding 1 x 10^5 cells per well in 24-well plates provides optimum confluence as shown in **Figure 1**.

Viability in cultured cells was lower in the steatogenic groups, Mild and Severe, compared with the control conditions. In fact, viability progressively diminished as time of the culture increased, reaching the lowest of 60% at 4 days in severe steatosis (**Figure 2A**). Accordingly, the mortality rate was higher in hepatocytes cultured in the steatogenic conditions, and it progressively increased with the time of exposure to lipids (**Figure 2B**). Cell numbers progressively increased as a result of proliferation (**Figure 2C**). However, proliferation rate was lower in Mild steatosis at 3 days and 4 days. In contrast, Severe steatosis was associated with lower proliferation from 24 h.

HepG2 cells cultured in the proposed protocol show the most important feature of steatosis, intracellular lipid accumulation. Staining cells with Oil Red O allowed to observe at least a two-fold increase of lipid droplets in cells cultured under steatogenic conditions as shown in **Figure 3** and **Figure 4**. Intracellular fat increased according to the time of exposure of culture in the steatogenic medium (**Figure 3**). In Mild steatosis, lipid contents were increased from day 2, whereas in Severe steatosis, they were significantly high from 24 h.

FIGURE AND TABLE LEGENDS:

Table 1. Hepatocyte culture in steatogenic conditions. The table presents the type of fatty acid used, the conditions maintained, and the observed outcomes in hepatocyte culture. PA: Palmitic acid. OA: Oleic acid.

Figure 1: Cell growth. HepG2 hepatocytes culture in control (**Figure 1A–D**) and mild steatogenic conditions (**Figure 1E–H**). Photographs show growth from 1–4 days of culture. Scale bar = 500 µm.

Figure 2: Viability and mortality rates. (**A**) Viability. (**B**) Mortality. (**C**) Cell number. HepG2 hepatocytes culture in control and steatogenic conditions were assessed for viability and mortality rates by trypan blue staining. Mean ± SD. Two independent experiments in triplicate per time of culture. Circles: control conditions; Squares: mild steatosis; Triangles: severe steatosis. One-way ANOVA was used to compare among conditions and time of culture for the same condition. $p < 0.05$ was considered significant: "*"- control vs severe steatosis; "§"- control vs mild steatosis; "¶"- mild vs severe steatosis.

Figure 3: Lipid accumulation. HepG2 hepatocytes culture in control and steatogenic conditions

were assessed for lipid contents by oil red O staining followed by a morphometric analysis using ImageJ software (NIH, USA). Percentage of lipids refers to the percentage of area stained by Oil-Red O (lipids) considering 100% as the complete area of each optical field analyzed. Mean ± SD. Two independent experiments in triplicate per time of culture. Circles: control conditions; Squares: mild steatosis; Triangles: severe steatosis. One-way ANOVA was used to compare among conditions and time of culture for the same condition. $p < 0.05$ was considered significant: "*"- control vs severe steatosis; "§"- control vs mild steatosis; "¶"- mild vs severe steatosis.

Figure 4: Steatosis *in vitro*. HepG2 hepatocytes culture in control (**Figure 4A–D**), mild steatogenic (**Figure 4E–H**), and severe steatogenic (**Figure 4I–L**) conditions were assessed for lipid contents by oil red O staining. Photographs show hepatocyte lipid droplets from 24 h to 4 days. Scale bar = 50 µm.

DISCUSSION:

This protocol is intended to provide a strategy to study steatosis *in vitro*. Cell culture is a powerful tool to study cellular, molecular, biochemical, and toxicological aspects of the cells exposed to different conditions. With this approach, steatosis can be visualized not only as a stage of the complex disease that is MAFLD, but also as the hepatocyte overexposure to lipids and the possible outcomes resulting from such exposure. Therefore, its application is not restricted to the physiopathology of MAFLD, but to the fact that patients with fatty liver are exposed to therapeutic drugs, contaminants, among other conditions that might be affected by steatosis. Thus, this protocol has potential applications in toxicology, pharmacology, and the identification of therapeutic targets for treatment of the disease.

On development of this protocol, one of the most critical steps is the preparation of the steatogenic mix: 1 part of palmitate: 2 parts of oleate, which induces steatosis from the day 2 (**Figure 3**, **Figure 4**), allowing hepatocytes to proliferate—despite a modest decrease in viability rate and increased mortality rate (**Figure 2**). However, decreased viability should not exceed 30%–40%, since that might represent a toxic effect rather than one that might be followed in the long-term. Hepatic steatosis is the result of a long-term overexposure to lipids. In this sense, lipids accumulate, at first, with mild affections on the hepatocytes, as observed in this model. Another feature is the lipid droplet profile. In mild steatosis, an increased size in lipid droplets is observed during the progression of the culture (**Figure 4E–H**). In severe steatosis, droplet size is considerably higher compared to mild steatosis (**Figure 4I–L**), whereas controls do not show changes in lipid droplet size (**Figure 4A–D**).

It is preferable to store both the mild and severe steatogenic media at 4 °C for up to a week. Afterward, it is recommended to prepare fresh steatogenic medium. However, the OA stock can be preserved at -20 °C for up to a month, whereas the PA stock can be stored at 4 °C for up to a month. Using these stock solutions after the suggested time might represent a risk of degradation of the fatty acids. To ensure the proper concentration of the solutions before every use, it is recommended to measure fatty acid concentrations by a non-esterified fatty acids (NEFA) assay kit.

PA and OA, as well as their respective salts, have been used separately to induce lipid accumulation; however, differences are observed for every fatty acid[16,20]. On one hand, palmitate used alone is a good inductor of steatosis. It induces cell death, hepatic insulin resistance, mitochondrial dysfunction, reticular stress[16,34–36]. However, palmitate is highly toxic[16,34], and the outcomes expected of using it alone in culture include lower viability and higher mortality compared with the mixture of PA and OA[16,20]. On the other hand, oleate also induces steatosis. It induces *de novo* lipogenesis, insulin resistance[37,38], and hyperproliferation[38]. However, the outcomes observed with oleate are often milder compared with palmitate and the mixture[16,20]. This might be related to its protective role. Oleate is the major component in olive oil, a key ingredient in the Mediterranean diet, one of the well-known successful strategies against MAFLD[39].

This protocol might be considered as a nice tool to study steatosis due to its reproducibility and the short time it takes to obtain results. This is in comparison with using experimental models of MAFLD, adding the fact that it does not imply the ethical issues inherent to using rodent models. This protocol allows being followed for several days, provided that steatogenic medium is refreshed every 24 h. This model is also cost-effective and possesses a wide range of applications. It can also be adjusted to other cell lines, not only hepatocytes, but a wide range of cells affected by lipid overexposure during obesity. One of the limitations of this protocol is the use of HepG2 cells. Since this is a carcinogenic cell line, it might conceal or increase some outcomes. However, the application of HepG2 cells in these types of studies is widely accepted due to its resemblance in lipid metabolism to healthy hepatocytes[40]. The use of the mixture PA:2OA might also prove to be controversial since it does not fully resemble the profiles of NEFA observed in the blood of NAFLD/MAFLD patients[41]. Other fatty acids, including linolenic or stearic acids, might be included in further modifications and improvements of this protocol. Another limitation is the fact that only one cell type, hepatocytes, is studied, lacking the interaction with other hepatic cells present in the liver, including sinusoidal endothelial, Kupffer, hepatic stellate cells, etc., that are engaged in the progression of MAFLD. Moreover, this is a model to induce steatosis exclusively, with no progression to steatohepatitis and fibrosis.

In conclusion, the study provides an *in vitro* protocol of hepatocyte steatosis that is easy to implement, reproducible, and with a wide range of applications in the study of steatosis as well as the hepatocyte function in the context of fatty liver.

ACKNOWLEDGMENTS:
This work was funded by Consejo Nacional de Ciencia y Tecnología (Conacyt, CB-221137). Adriana Campos is a doctoral student at Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, and was supported by Conacyt (CVU: 1002502).

DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:

1. Younossi, Z. M. Non-alcoholic fatty liver disease - a global public health perspective. *Journal of Hepatology*. **70** (3), 531–544 (2019).
2. Younossi, Z. et al. Global perspectives on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. *Hepatology*. **69** (6), 2672–2682 (2019).
3. Eslam, M. et al. A new definition for metabolic dysfunction-associated fatty liver disease: An international expert consensus statement. *Journal of Hepatology*. **73** (1), 202–209 (2020).
4. Eslam, M., Sanyal, A. J., George, J. MAFLD: A consensus-driven proposed nomenclature for metabolic associated fatty liver disease. *Gastroenterology*. **158** (7), 1999–2014 e1991 (2020).
5. Chalasani, N. et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American association for the study of liver diseases. *Hepatology*. **67** (1), 328–357 (2018).
6. Calzadilla Bertot, L., Adams, L. A. The natural course of non-alcoholic fatty liver disease. *International Journal of Molecular Science*. **17** (5), 774 (2016).
7. Reccia, I. et al. Non-alcoholic fatty liver disease: A sign of systemic disease. *Metabolism*. **72**, 94–108 (2017).
8. Tomita, K. et al. Free cholesterol accumulation in hepatic stellate cells: Mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice. *Hepatology*. **59** (1), 154–169 (2014).
9. Byrne, C. D., Targher, G. Nafld: A multisystem disease. *Journal of Hepatology*. **62** (1 Suppl), S47–64 (2015).
10. Ipsen, D. H., Lykkesfeldt, J., Tveden-Nyborg, P. Molecular mechanisms of hepatic lipid accumulation in non-alcoholic fatty liver disease. *Cellular and Molecular Life Sciences*. **75** (18), 3313–3327 (2018).
11. Diehl, A. M., Day, C. Cause, pathogenesis, and treatment of nonalcoholic steatohepatitis. *New England Journal of Medicine*. **377** (21), 2063–2072 (2017).
12. Zeng, X. et al. Oleic acid ameliorates palmitic acid induced hepatocellular lipotoxicity by inhibition of ER stress and pyroptosis. *Nutrition and Metabolism (London)*. **17**, 11 (2020).
13. Ore, A., Akinloye, O. A. Oxidative stress and antioxidant biomarkers in clinical and experimental models of non-alcoholic fatty liver disease. *Medicina (Kaunas)*. **55** (2), 26 (2019).
14. Paradies, G., Paradies, V., Ruggiero, F. M., Petrosillo, G. Oxidative stress, cardiolipin and mitochondrial dysfunction in nonalcoholic fatty liver disease. *World Journal of Gastroenterology*. **20** (39), 14205–14218 (2014).
15. Aravinthan, A. et al. Hepatocyte senescence predicts progression in non-alcohol-related fatty liver disease. *Journal of Hepatology*. **58** (3), 549–556 (2013).
16. Gomez, M. J. et al. A human hepatocellular in vitro model to investigate steatosis. *Chemico Biological Interactions*. **165** (2), 106–116 (2007).
17. Wu, W. K. K., Zhang, L., Chan, M. T. V. Autophagy, NAFLD and NAFLD-related HCC. *Advances in Experimental Medicine and Biology*. **1061**, 127–138 (2018).

18. Levy, G., Cohen, M., Nahmias, Y. In vitro cell culture models of hepatic steatosis. *Methods in Molecular Biology*. **1250**, 377–390 (2015).
19. Kanuri, G., Bergheim, I. In vitro and in vivo models of non-alcoholic fatty liver disease (NAFLD). *International Journal of Molecular Science*. **14** (6), 11963–11980 (2013).
20. Ricchi, M. et al. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *Journal of Gastroenterology and Hepatology*. **24** (5), 830–840 (2009).
21. Lee, Y. et al. Serial biomarkers of de novo lipogenesis fatty acids and incident heart failure in older adults: The cardiovascular health study. *Journal of the American Heart Association*. **9** (4), e014119 (2020).
22. Alnahdi, A., John, A., Raza, H. Augmentation of glucotoxicity, oxidative stress, apoptosis and mitochondrial dysfunction in Hepg2 cells by palmitic acid. *Nutrients*. **11** (9), 1979 (2019).
23. Chen, X. et al. Oleic acid protects saturated fatty acid mediated lipotoxicity in hepatocytes and rat of non-alcoholic steatohepatitis. *Life Sciences*. **203**, 291–304 (2018).
24. Xing, J. H. et al. NLRP3 inflammasome mediate palmitate-induced endothelial dysfunction. *Life Sciences*. **239**, 116882 (2019).
25. Yan, H. et al. Insulin-like Growth Factor Binding Protein 7 accelerates hepatic steatosis and insulin resistance in non-alcoholic fatty liver disease. *Clinical and Experimental Pharmacology and Physiology*. **46** (12), 1101–1110 (2019).
26. Wang, J., Hu, R., Yin, C., Xiao, Y. Tanshinone IIA reduces palmitate-induced apoptosis via inhibition of endoplasmic reticulum stress in Hepg2 liver cells. *Fundamental and Clinical Pharmacology*. **34** (2), 249–262 (2020).
27. Xiao, Z., Chu, Y., Qin, W. IGFBP5 modulates lipid metabolism and insulin sensitivity through activating ampk pathway in non-alcoholic fatty liver disease. *Life Sciences*. **256**, 117997 (2020).
28. Avila, G. et al. In vitro effects of conjugated linoleic acid (CLA) on inflammatory functions of bovine monocytes. *Journal of Dairy Science*. **103** (9), 8554–8563 (2020).
29. Malhi, H., Bronk, S. F., Werneburg, N. W., Gores, G. J. Free fatty acids induce JNK-dependent hepatocyte lipoapoptosis. *The Journal of Biological Chemistry*. **281** (17), 12093–12101 (2006).
30. Oh, J. M. et al. Effects of palmitic acid on TNF-alpha-induced cytotoxicity in SK-Hep-1 cells. *Toxicology In Vitro: An International Journal Published in Association with BIBRA*. **26** (6), 783–790 (2012).
31. Stellavato, A. et al. In vitro assessment of nutraceutical compounds and novel nutraceutical formulations in a liver-steatosis-based model. *Lipids in Health and Disease*. **17** (1), 24 (2018).
32. Pachikian, B. D. et al. Implication of trans-11, trans-13 conjugated linoleic acid in the development of hepatic steatosis. *PLoS One*. **13** (2), e0192447 (2018).
33. Ferreira, T. Rasband, W. Analyze in *ImageJ User Guide*. Ch. 30, 132–135 (2012).

34. Geng, Y., Wu, Z., Buist-Homan, M., Blokzijl, H., Moshage, H. Hesperetin protects against palmitate-induced cellular toxicity via induction of GRP78 in hepatocytes. *Toxicology and Applied Pharmacology*. **404**, 115183 (2020).
35. Geng, Y. et al. Protective effect of metformin against palmitate-induced hepatic cell death. *Biochimica et Biophysica Acta - Molecular Basis of Disease*. **1866** (3), 165621 (2020).
36. Sarnyai, F. et al. Effect of cis- and trans-monounsaturated fatty acids on palmitate toxicity and on palmitate-induced accumulation of ceramides and diglycerides. *International Journal of Molecular Science*. **21** (7), 2626 (2020).
37. Chen, J. W. et al. Tetrahydrocurcumin ameliorates free fatty acid-induced hepatic steatosis and improves insulin resistance in HepG2 cells. *Journal of Food and Drug Analysis*. **26** (3), 1075–1085 (2018).
38. Burhans, M. S. et al. Hepatic oleate regulates adipose tissue lipogenesis and fatty acid oxidation. *Journal of Lipid Research*. **56** (2), 304–318 (2015).
39. Abenavoli, L., Milanovic, M., Milic, N., Luzza, F., Giuffre, A. M. Olive oil antioxidants and non-alcoholic fatty liver disease. *Expert Reviews in Gastroenterology and Hepatology*. **13** (8), 739–749 (2019).
40. Nagarajan, S. R. et al. Lipid and glucose metabolism in hepatocyte cell lines and primary mouse hepatocytes: A comprehensive resource for in vitro studies of hepatic metabolism. *American Journal of Physiology - Endocrinology and Metabolism*. **316** (4), E578–E589 (2019).
41. Hodson, L., Skeaff, C. M., Fielding, B. A. Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Progress in Lipid Research*. **47** (5), 348–380 (2008).

Figure1

Click here to access/download;Figure;Figure 1.jpg

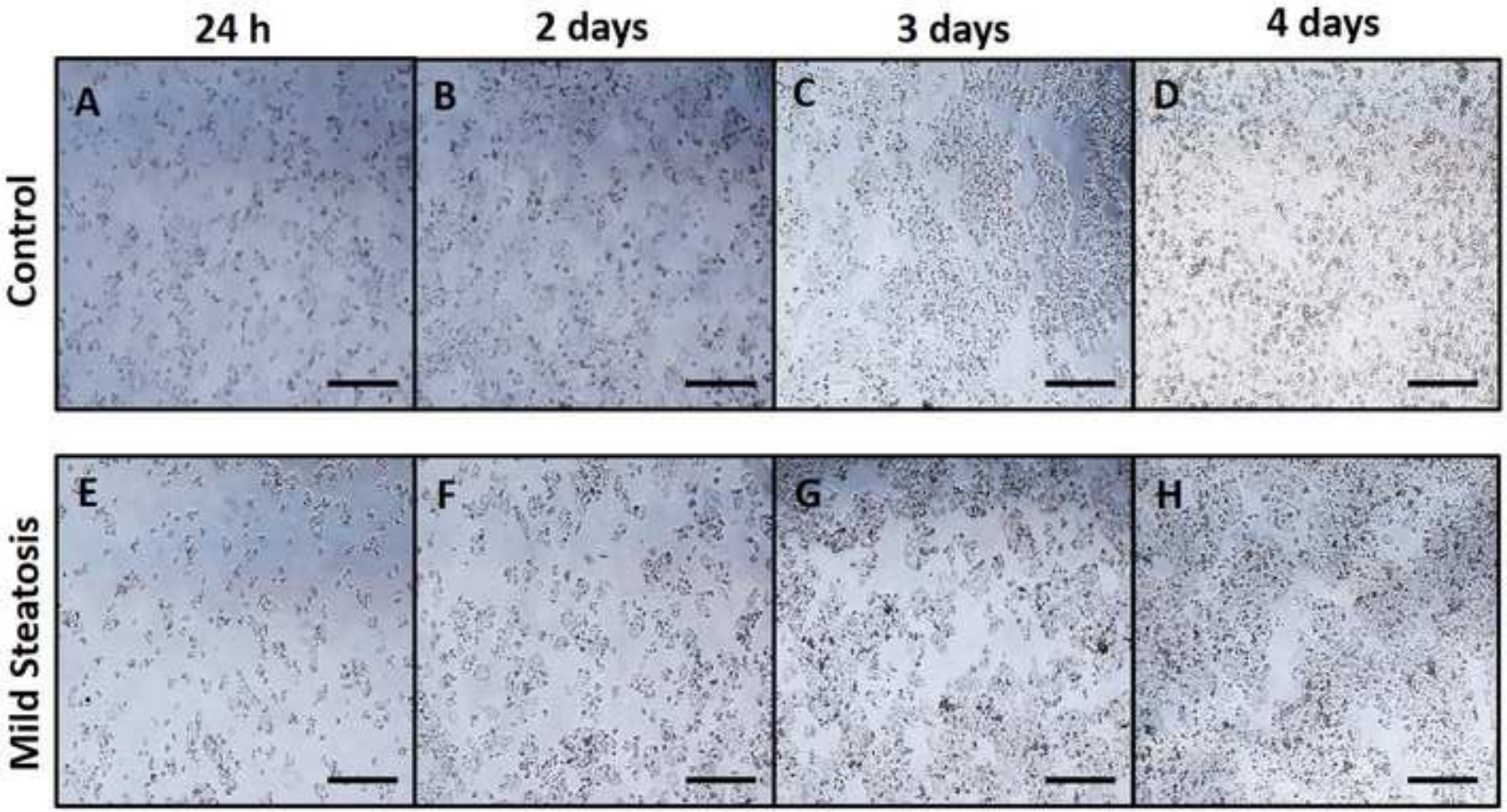

Figure2

Click here to access/download;Figure;Figure 2.jpg

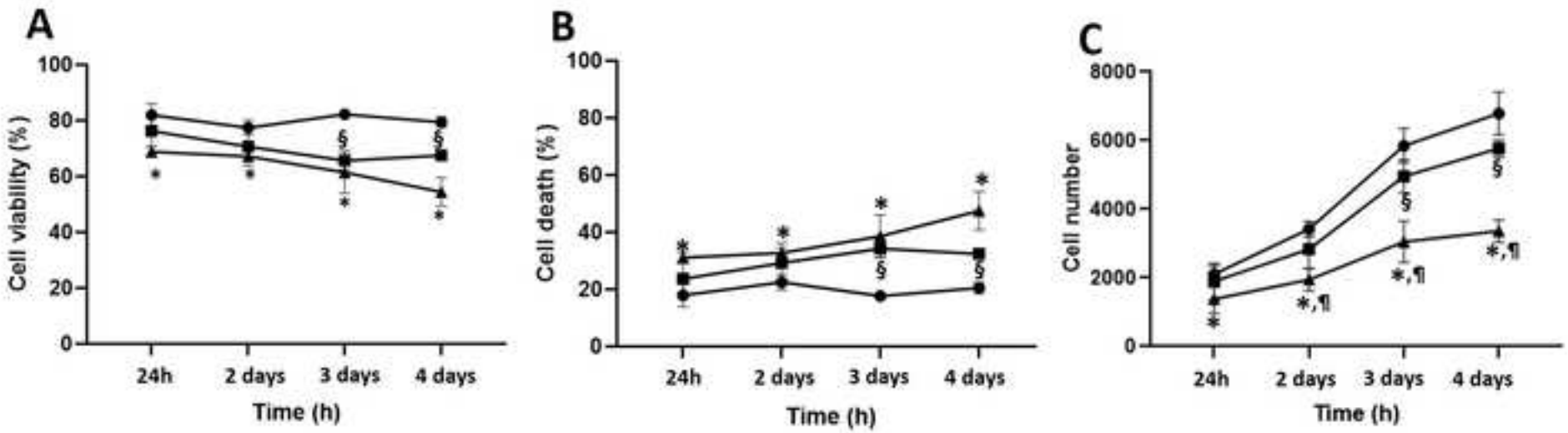

Figure3

Click here to access/download;Figure;Figure 3.jpg

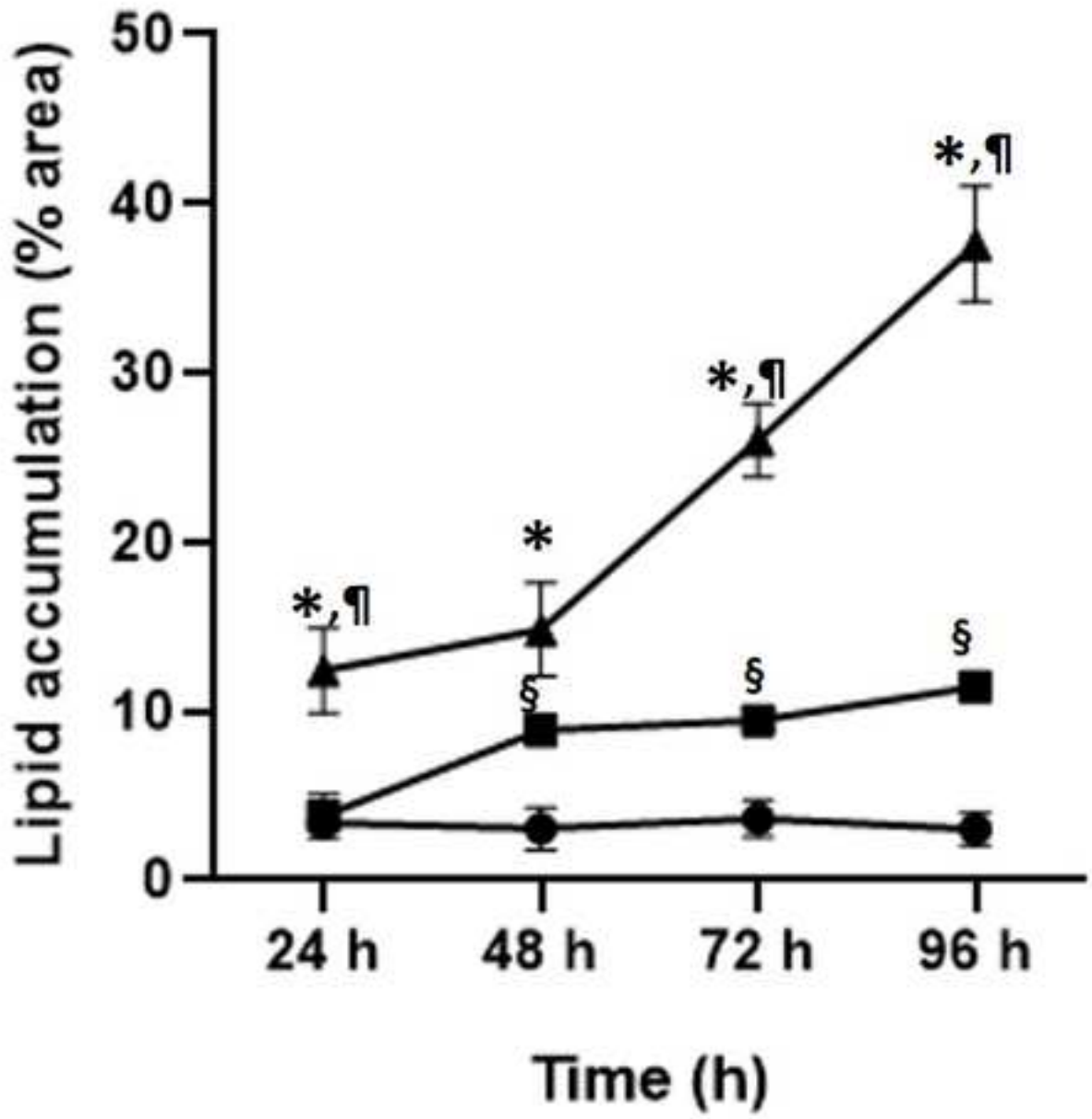

Figure4

Click here to access/download;Figure;Figure 4.jpg

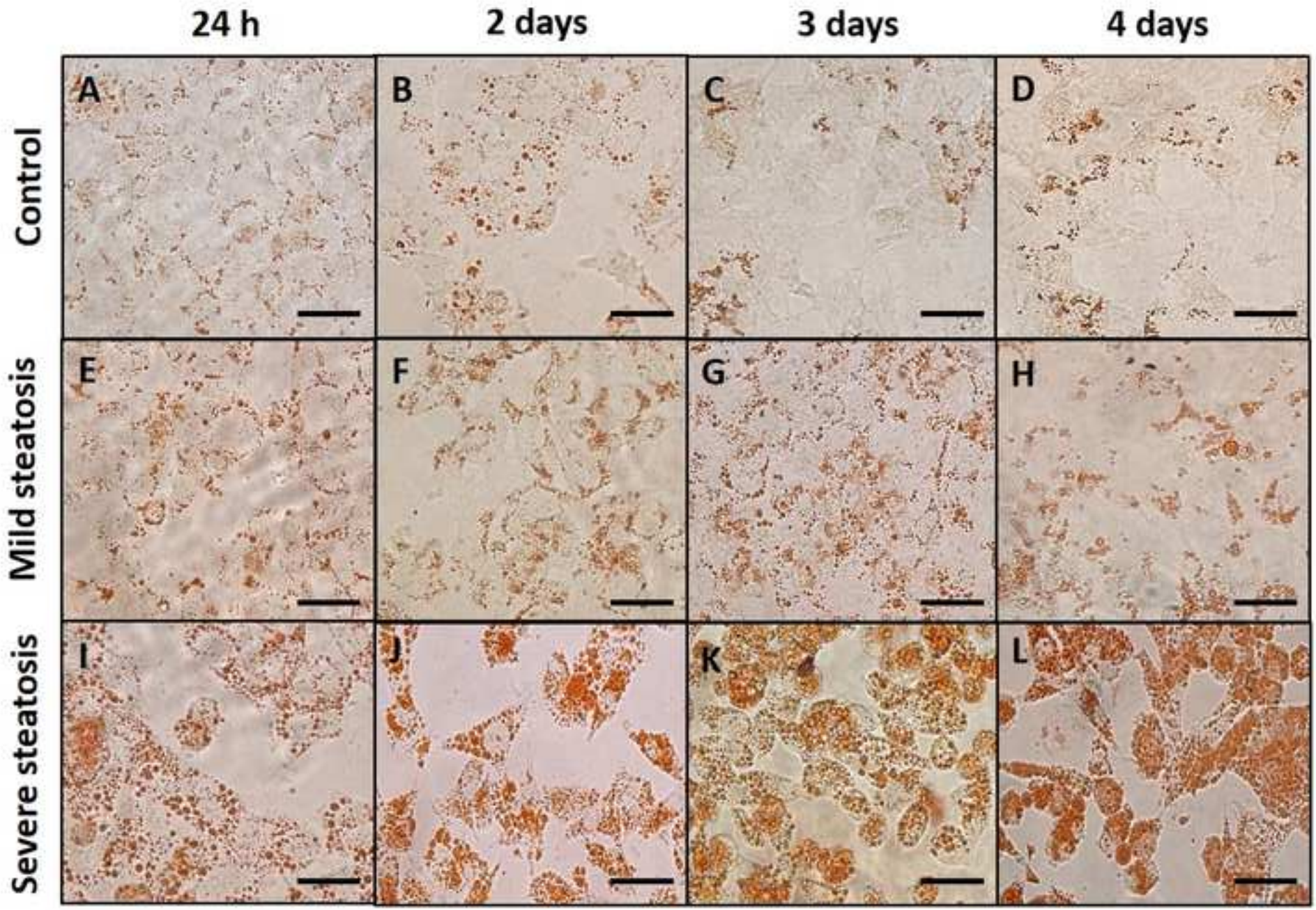

Table 1. Hepatocyte culture in steatogenic conditions

Fatty acids	Conditions
PA	Concentration: 200 µM
	Time exposition: 24 h
PA	Concentration: 50, 100 and 200 µM
	Time exposition: 24 h
PA	Concentration: 250 µM , 500 µM , 750 µM and 1,000 µM
	Time exposition: 24 h
Mix of OA/PA	Concentration: 1 mM
	Time exposition: 24 h
	Rate: 2OA:1PA
Mix of OA/PA	First stimulation with 200 µM and 400 µM of PA and then second stimulation with 200 µM of OA
	Concentration:400 µM PA: 200 µM OA
	Rate: 2PA:1OA
	Time exposition: 24 h
Mix of OA/PA	Concentration: 400 µM PA: 200 µM OA
	Rate: 2PA:1OA
	Time exposition: 24 h
Mix of OA/PA	Concentration :50 and 500 µM
	Rate: 2PA:1OA
	Time exposition: 24 h, 2 days,3 days and 4 days.

Outcomes	Reference
Lipid accumulation	Yan et al, 2019[25].
Hepatocyte damage	
Transaminases elevation	
Lipid accumulation	Xing et al, 2019[24].
Lipid accumulation	Wang et al, 2020[26].
Progressive reduction of cell viability	
Lipid accumulation	Xiao et al, 2020[27].
Does not report lipotoxicity	
Lipid accumulation.	Zeng et al, 2020[12].
Evidence of lipotoxicity induced by PA was reduced by stimulation of OA.	
Lipid accumulation	Chen et al, 2018[23].
Generation of two types of steatosis: mild steatosis and severe s teatosis.	Campos and Guzmán 2021
Simulates chronic exposition of lipid overload	

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Biosafety cabinet	ESCO Airstream	AC2-452+C2:C26
Bottle top filter	Corning, US	430513
Bovine serum albimun (BSA)	Gold Biotechnology, US	A-421-10
Culture media RPMI 1640	ThermoFisher-Gibco, US	31800-022
Fetal Bovine Serum (FBS)	ThermoFisher-Gibco, US	A4766801
Hemocytometer	Marienfeld, DE	640010
HepG2 cell line	ATCC, US	HB-8065
Humidified incubator	Thermo Electronic Corporation,	Model: 3110
Inverted microscope Eclipse	NIKON, JPN	Model: TE2000-S
Isopropanol	Sigma-Aldrich, US	I9030-4L
Oil Red O Kit	Abcam, US	ab150678
Paraformaldehyde	Sigma-Aldrich, US	P6148-500G
Penicillin/streptomycin	ThermoFisher-Gibco, US	15140-122
pH meter	Beckman, US	Model: 360 PH/Temp
Phosphate buffered saline	ThermoFisher-Gibco, US	10010-023
Serological Pipettes	Sarstedt, AUS	86.1253.001
Serological Pipettes	Sarstedt, AUS	86.1254.001
Sodium bicarbonate	Sigma-Aldrich, US	S5761-1KG
Sodium oleate	Santa Cruz Biotechnology, US	sc-215879A
Sodium palmitate	Santa Cruz Biotechnology, US	sc-215881
Syring filter	Corning, US	431219
Trypan Blue	Sigma-Aldrich, US	T6146-25G
Trypsin 0.05% /EDTA 0.53 mM	Corning, US	25-052-Cl
24 well cell culture cluster	Corning, US	3524
96 well cell culture cluster	Corning, US	3599

Comments/Description
Class II Type A2 Biological Safety Cabinet
Non-pyrogenic, polystyrene, sterile. 1 filter/Bag. 0.22 µm, 500 mL.
BSA Fatty Acid Free for cell culture
-
-
-
Hepatocellular carcinoma human cells.
Temperature (37 °C ± 1 °C), humidity (90% ± 5%) , CO_2 (5% ± 1%)
-
-
Kit for histological visualization of neutral fat.
-
Antibiotics 10,000 U/mL Penicillin, 10,000 µg/mL Streptomycin
-
-
Non-pyrogenic, sterile, 5 mL
Non-pyrogenic, sterile, 10 mL
Preparation of culture media
-
-
Non-pyrogenic, sterile, 28 mm, 0.2 µm.
-
-
Flat bottom with lid. Tissue culture treated. Nonpyrogenic, polystyrene, sterile. 1/Pack.
Flat bottom with lid. Tissue culture treated. Nonpyrogenic, polystyrene, sterile. 1/Pack.

Dear Dr. Amit Krishnan,

We would like to take this opportunity to thank you and the Reviewers for your kind consideration and contributions to the improvement of our manuscript. Please find the answers to every comment below.

Best regards,

Carolina Guzmán, PhD.

Editorial comments:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have proofread the manuscript and corrected the spelling and grammar issues detected.

2. Line 224-226: Please specify whether there is a PBS wash before adding Oil red solution.

There is no need for a PBS wash, now it has been included in line 231

3. Line 226-228: Please specify if the oil-red solution is discarded before rinsing with 1 mL of distilled water.

In fact, excess of oil-red O must be discarded. Now it is included in line 235

4. Line 230-232: Please specify if the hematoxylin solution is discarded before rinsing with 1 mL of distilled water.

Excess of hematoxylin needs to be discarded before rising, it has been specified in line 241

5. Line 234: Please include the details of magnification.

In this case is recommended to observe at 400 magnification: Objective 40x, Ocular 10x. These details have been included in line 245

6. Line 241-242: Please include more details to the step. How is the assessment performed? A citation would suffice.

We have referred to the Ferreira T, and Rasband W, ImageJ user guide in the text. Please find this reference in line 252-253 and in the reference list number 33.

7. Line 244-246: How is the comparison done. Is it compared using ImageJ software or manually using some other application?

This comparison is performed by using ImageJ, it is now specified in the protocol and referred to the ImageJ user guide. Please see line 255-256, reference 33.

8. Line 322-324: Please ensure that the sentence is correct. Consider breaking it into two sentences.

We split the sentence into two and rearrange the resulting second sentence. Please see line 338-341

9. Table 1: Please include a Table Legend for Table 1 in the Figure and Table Legend Section

The Table Legend for Table 1 has been included in line 283-284

10. Figure 2: The legend for Figure 2 discusses only 2A and 2B. Please include a description for 2C.

We apologize for this. Now we include the description of Figure 2C (line 290)

11. Figure 2/3: Please include the description for the symbols "¶" and "§" in the Figure and Table Legends of the respective figures.

We have included the descriptions por symbols "¶" and "§". Please find them in lines 295-296 and 305

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The revised manuscript by Campos-Espinonsa and Guzmán utilised a lipid loading protocol to provide an easily accessible cellular model to investigate hepatic steatosis. The authors optimised a lipid medium containing 2:1 oleate:palmitate to induce steatosis, which was measured using intracellular lipid content via Oil-Red-O and viability in HepG2 cells. In the revised version, authors have added an additional level of steatosis to now have 1) mild and 2) severe steatosis. The authors have also commented on changes in lipid droplet profile which is important for phenotyping NAFLD. The overall changes made are satisfactory and there are no major concerns. Minor concerns outlined below are ways to improve the manuscript to be more reflective of the emerging lipid community.

We thank the reviewer for the kind comments and contributions to the improvement of our manuscript.

Minor Concerns:
Technical
1. There is currently no table legend for Table 1.

We have included the table legend. Please find it in line 283-284

2. Change 'n' statements to reflect n number. Right now it says n = 3 wells in 2 independent experiments which is misleading as it is n = 2 not n =3 (i.e. n = 2 independent experiments with 3 wells each or in triplicate).

We have replaced the n statements as suggested (line 292-293 and 302)

3. As another level of steatosis has been added, Figure 1 label should read 'mild steatosis' for better clarity and this should be reflected in the figure legend as well.

We have corrected both Figure 1 and its Figure Legend to indicate Mild steatosis.

4. For table 1, since data from individual papers are outlined, it is better to write out the author references (e.g. Author et al 2009+ref number) instead of just the ref number

We have included the Authors in Table 1

Text
1. When discussing Table 1, need to be clear that this is only a table of selected studies where specifically oleate, palmitate or both were used. It is worth mentioning in the text that other fatty acids/FA mixtures have also been used in in vitro studies, but that this table reflects studies that use the 2 most abundant fatty acids in hepatocytes.

We have included information regarding other fatty acids used in in vitro studies as suggested. Please referred to lines 86-91

2. I'm still not convinced that 2:1 oleate:palmitate is completely reflective of human plasma NEFAs. The authors mentioned that palmitate (SFA) and oleate (MUFA) make up ~1/3 each (which is true - 28.3% palmitate, 32.7% oleate from Hodson et al 2008 Prog Lip Res), but the reasoning that oleate is a suitable stand-in for the last 1/3 of FAs in human plasma is debatable. This is particularly clear when considering that 13.5% of human plasma NEFA is linoleic acid alone (a PUFA). I do not think this detracts from the value of the protocol, but it should be listed as part of the limitations to consider.

We have now acknowledged this limitation in the discussion (line 364-367)

Reviewer #2:
Manuscript Summary:
Authors presented a protocol of culturing hepatocytes in high lipid condition, which can be useful in steatosis research.

We thank the reviewer for the kind comments and contributions to the improvement of our manuscript.