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Title: Silybin counteracts lipid excess and oxidative stress in cultured steatotic hepatic cells

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Dear Editor,
thank you for your letter. We appreciated very much the comments and suggestions of the reviewers and we corrected the manuscript according to their requirements.
Enclosed you will find the responses to the specific comments. In the revised version of our manuscript, the changes have been indicated using red bold characters.

Thank you very much for your attention

Yours sincerely

Laura Vergani

REVIEWER 1

We are grateful to the reviewer whose suggestions allowed us to clarify some points and further improve our manuscript. Moving to the specific comments, we are pleased to provide a point-to-point reply:

- *Introduction is too long and silybin has presented to the reader in the very end of the introduction. Introduction must be rewritten to better demonstrates the possible interaction between silybin in NASH.*

We agree with the criticism and we shortened Introduction. As suggested by the reviewer we moved up the paragraph regarding silybin and we better discussed the possible association between silybin and NASH.

- *Down-regulated oxidative stress promoted by silybin can signal to FA catabolism?*

As emphasized in the manuscript, our study shows that the reduction in fat accumulation exerted by silybin in the steatotic hepatocytes is associated with the improvement of the oxidative stress caused by lipid excess.

- *Please include studies and/or experiments demonstrating oxidative stress may modulate PPARs.…*

Little is known about the association between oxidative stress and PPARs. We briefly discussed the results of Teissier et al regarding connections between PPARs, LDL metabolism, and oxidative stress. In fact, Teissier found that synthetic PPAR agonists induce the production of reactive oxygen species (ROS) by inducing NADPH oxidase.

- *Up-regulation of PPARα and down-regulation of PPARg mRNA levels promoted by silybin treatment, only suggests that silybin is associated with a stimulation of mitochondrial oxidation. Please, provide studies and/or experiments demonstrating oxidative up-regulation of PPARα and down-regulation of PPARg mRNA are related to increased oxygen consumption and of mitochondrial oxidation.*

We agree with the criticism that our data on stimulation of mitochondrial oxidation by silybin are at moment mainly indirect. Experiments of oximetry are in progress to assess the effects of silybin on mitochondrial activity and on respiratory chain enzymes, and will be object of a next paper. However, to better clarify this aspect, in the manuscript, we discussed with more details the

converging results which suggest a stimulation of fat catabolism as a consequence of silybin exposure. In fact not only the up-regulation of PPARα and the down-regulation of PPARγ seem to indicate this effect, but also the up-regulation of CPT1. Indeed, CPT1 is the first component and rate-limiting step of mitochondrial β–oxidation as it allows long chain fatty acids (LCFA) to enter the mitochondria. Many studies indeed indicate that the activity of CPT1 determines the rate of FA oxidation and that over-expression of CPT1a in cultured cells increases fatty acid oxidation.

REVIEWER 2

We thank the reviewer for his positive comments to our work. The text has been modified accordingly to the requests.

- *They used hepatome FaO cell as model to monitor, how about normal hepatocyte*?

In the last decades, *in vitro* models have been developed to investigatee the mechanisms involved in the progression of NAFLD. Exposure of hepatocytes to high concentrations of FAs *in vitro* resulted in lipid overload similar to the steatosis observed in both patients and animal models with NAFLD. Both primary cell cultures and immortalized cell lines of hepatocytes can be used to develop *in vitro* models of NAFLD. Both ethical issues and limited number of human liver biopsies make it complicated to use primary human cell cultures. An alternative are primary rodent cells, but methods used for isolation need to be guarantee reproducible results, and sometimes cells loose tissue-specific functions when cultured for a long time. An alternative to primary cell cultures are immortalized cell lines, which have high replicative capacity, and a stable phenotype. In addition, cultivation of immortalized cell lines is simpler and easier to standardize (for overview see Chavez-Tapia, 2011). Moreover, the FaO cell line used in this study is a liver cell line maintaining hepatocyte specific markers as stated in the Materials and Methods paragraph.

- *… PPAR mRMA show different expression, how about protein level for PPAR?*

The studies at the mRNA level are largely based on a key assumption that mRNA expression is informative in predicting protein expression level. Several results showed an overall positive correlation between mRNA and protein expression levels, even though it is certainly far from perfect. In general, data on the transcription level can suggest whether a specific protein is present and/or it has changed its level as a function of the treatment. Therefore, transcription data are useful for identifying potential candidates for follow-up work at the protein level. However, we can

underline that our data showing a modulation of PPAR transcription as a response to exposure of cells to FAs and/or silybin allow to identify which pathways are switch on/off at short times thus giving information on the dynamic of the response.

- *… Can they show silybin alone in FaO cell viability?*

We added a sentence about the null effect of silybin on cell viability in Materials and Methods section.