

Use of an *in vitro* model of hepatic steatosis for studying the anti-oxidant and anti-steatotic effects of fucoidan polysaccharides

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we aimed to investigate the anti-oxidant and anti-steatotic effects of FUs purified from *C. compressa*, *F. hermonis*, and *E. globulus*. To this aim, we used a validated NAFLD *in vitro* model consisting of rat hepatoma FaO cells exposed to an oleate/palmitate mixture. Such a model is suitable for rapid investigation of direct effects of natural and artificial compounds, together with satisfying the strategy of 3Rs for laboratory use of animals. Our results indicated that all FUs display anti-oxidant and anti-steatotic activities. Steatotic FaO cells may be employed to further study the biological effects of FUs.

Introduction

Non Alcoholic Fatty Liver Disease (NAFLD) is the most common cause of liver disorders with high negative impact on human health. It is characterized by fat accumulation in more than 5% of hepatocyte, stored in the form of triacylglycerols (TAGs) within cytosolic lipid droplets (LDs), as a result of hepatic lipid metabolism imbalance. Molecular mechanisms and therapeutic strategies for NAFLD can be studied using *in vivo* models, which offer

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Abstract

Non Alcoholic Fatty Liver Disease (NAFLD) is characterised by fat accumulation in hepatocytes in the form of triacylglycerols (TAGs) within cytosolic lipid droplets. Fucoidans (FUs) are biologically active polysaccharides usually isolated from brown marine algae, but recently identified also in terrestrial plants. In this study,

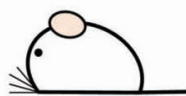
the advantage to study the effects of active compounds at the level of the whole organism. However, major disadvantages are ethical concerns, requirement of skilled manpower, time consuming protocols and high costs, thus supporting the application of *in vitro* models which may satisfy the strategy

In vivo NAFLD model:
High Fat Diet (HFD)-fed rats



5 animals/group
1 wk acclimatization
5 wks of HFD to achieve steatosis

In vitro NAFLD model:
rat hepatocyte primary cultures



1 animal
24 hrs for cell seeding and attachment
24 hrs of OP treatment to achieve steatosis

In vitro NAFLD model:
FaO rat hepatoma cell line



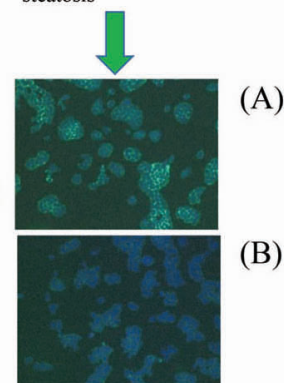
0 animal
3 hrs of OP treatment to achieve steatosis

Intracellular TAG content
(% of Ctrl)

Ctrl	100	
OP	275 ± 37	###
CYS	176 ± 21	##, **
FER	175 ± 10	##, **
EUC	120 ± 20	***

Data are mean ± SD, n = 3
(###p≤0.001; ##p≤0.01 vs Ctrl)
(***p≤0.001; **p≤0.01 vs OP)

24 hrs of incubation with
50 µg/mL FUs.



Bodipy/DAPI staining for
LDs detection in OP cells (A)
with respect to Ctrl (B)

Figure 1. Schematic description of different *in vivo* and *in vitro* NAFLD experimental models with reference to the 3R strategy; the anti-steatotic effects of FUs extracted from CYS, FER and EUC is also shown.

of 3 Rs (*i.e.* reduction, refinement and replacement) for laboratory use of animals.¹ In the last years our group used both *in vivo* and *in vitro* models to study NAFLD, and demonstrated that the main markers of hepatic steatosis are maintained in all the experimental models, thus making the *in vitro* model suitable for rapid investigation of direct anti-steatotic effects of natural and artificial compounds. In the present study, we used an *in vitro* model of NAFLD to study the anti-oxidant and anti-steatotic effects of fucoidans (FUs). FUs are fucose rich polysaccharides predominantly found in the cell wall of brown algae such as *Cystoseira compressa*, but recently identified also in terrestrial plants such as *Eucalyptus globulus* and *Ferula hermonis*, growing in Lebanon.

Materials and Methods

FUs were purified from *C. compressa* (CYS), *F. hermonis* (FER), and *E. globulus* (EUC) as previously described.² Anti-oxidant activity was determined by DPPH test. Steatotic rat hepatoma FaO cells were obtained by 3 h exposure to an oleate/palmitate (OP) mixture and then exposed to 50 µg/mL FUs for 24 h.^{1,3} The anti-steatotic effect of FUs was investigated by measuring intracellular TAG content and by detecting LD formation through fluorescence microscopy. The expression of PPAR (Peroxisome Proliferator Activated Receptor) isoforms and LD associated perilipins (PLINs) that play important roles in lipid homeostasis, was evaluated by qPCR.³

Results

The upper part of Figure 1 shows that a dramatic decrease in animal use and a high speeding up of the experimental procedures can be appreciated when comparing the number of animals needed and the duration of the treatments used *in vivo* and *in vitro* to obtain three different models of NAFLD. The lower part of Figure 1 shows the detection of LDs by Bodipy fluorescent staining in OP-treated FaO cells with respect to control (Ctrl) cells; the table shows that intracellular TAG content was significantly reduced in steatotic cells upon any FU treatment. This result was in line with a suppression of lipogenic genes such as PPAR γ , PLIN2 and PLIN5, which was measured by qPCR. DPPH assay allowed to demonstrate a significant anti-oxidant activity, which was exerted by FUs purified from all three species (IC 50 was 158.5 ± 1.81 , 152.9 ± 3.30 , 4.466 ± 3.26 µg/mL for CYS, FER and EUC, respectively).

Of note, FUs purified from terrestrial plants exerted stronger anti-oxidant and anti-steatotic effects than those obtained from marine brown algae, with *E. globulus* FUs showing the highest activities.

Conclusions

Different *in vivo* and *in vitro* models have been established to resemble the most important features of NAFLD. *In vitro* approaches have the advantage of providing a simple and highly reproducible model,

where the mechanisms can be studied directly at the cellular level. Our results validate the use of cell cultures as effectual tools to replace the use of laboratory animals when studying NAFLD and the effects and mechanisms of action of natural compounds with therapeutic potential, such as FUs. Steatotic FaO cells may be employed to further study the biological effects of FUs, particularly those extracted from *E. globulus*, which is confirmed as a powerful medicinal plant.

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