Quercetin and Hydroxytyrosol as modulators of hepatic steatosis: a NAFLD-on-a-chip study.

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Abstract

Organs-on-chip are increasingly catching on as a promising and valuable alternative to animal models, in line with the 3Rs initiative, to create 3D tissue microenvironments in which cells behave physiologically and pathologically at unparalleled precision and complexity. Indeed, these platforms offer new opportunities to model human diseases and test the potential therapeutic effect of different drugs as well as their limitations, overtaking the limited predictive accuracy of conventional 2D culture systems. Here, we present a liver-on-a-chip model to investigate the effects of two naturally occurring polyphenols, namely Quercetin and Hydroxytyrosol, on non-alcoholic fatty liver disease (NAFLD) using a method of high-content analysis. NAFLD is currently the most common form of chronic liver disease, whose complex pathogenesis is far from being clear. Besides, no definitive treatment has been established for NAFLD so far. In our experiments, we observed that both polyphenols seem to restrain the progression of the free fatty acid-induced hepatocellular steatosis, showing a cytoprotective effect due to their antioxidant properties. In conclusion, the resulting insights of the present work could guide novel strategies to contrast the onset and progression of NAFLD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide, which occurs in individuals who deny significant alcohol consumption (Abd El-Kader & El-Den Ashmawy, 2015). NAFLD encompasses a broad spectrum of liver pathologies ranging from simple steatosis (a benign enhanced fatty infiltration within the liver) to the more severe nonalcoholic steatohepatitis (NASH), characterized by inflammation, fibrosis and hepatocellular damage (ballooning), which may progress to fibrosis and end-stage liver disease, including cirrhosis and hepatocellular carcinoma (HCC) (Abd El-Kader & El-Den Ashmawy, 2015; Angulo, 2002; Starley, Calcagno, & Harrison, 2010). However, the multifactorial pathogenesis of NAFLD has not yet been fully understood, and highly effective therapeutics are still far from being available. The majority of hepatocellular lipids are stored as triglycerides (TGs), but other lipid metabolites, such as free fatty acids (FFAs) and cholesterol, may play a role in the development of the disease through the damaging effect of lipotoxicity (Abd El-Kader & El-Den Ashmawy, 2015; Feldstein et al., 2004). Intrahepatic lipid accumulation (i.e., excessive TGs content) and oxidative stress, in terms of generation of reactive oxygen species (ROS) and lipid peroxidation, are two of the main mechanisms involved in the etiology of liver diseases, including NAFLD, in which they play a crucial role in the onset and progression (Abd El-Kader

& El-Den Ashmawy, 2015; Adachi & Ishii, 2002). Interestingly, some recent studies, which used *in vitro* models of hepatic steatosis, showed the protective effect of two natural polyphenols, namely Hydroxytyrosol and Quercetin, against the development of NAFLD and in the reduction of intrahepatic lipogenesis (Hur et al., 2012; Priore, Siculella, & Gnoni, 2014; Vidyashankar, Sandeep Varma, & Patki, 2013). Two of these reports observed a beneficial effect of Oleuropein and its metabolite HT (the main polyphenols present in green olives, olive leaves and extra virgin olive oil) on lipogenesis and hepatic lipid synthesis, using mouse and human hepatoblastoma cell lines as well as primary-cultured rat hepatocytes (Hur et al., 2012; Priore et al., 2014). Similarly, Quercetin (a polyphenolic flavonoid compound from dietary origin and with known antioxidant and hypolipidemic activities) has been shown to attenuate and contrast NAFLD symptoms by reducing intrahepatic TG accumulation, oxidative stress and inflammatory cytokine production caused by oleic acid-induced hepatic steatosis in HepG2 cells (Vidyashankar et al., 2013).

Despite these findings, the traditional *in vitro* models of hepatic steatosis are not suitable for recreating the entire liver morphology and thus investigating the pathogenesis of NAFLD, as they rely on 2D cell culture monolayers that do not accurately reproduce the 3D physiological microenvironment of hepatic tissue; as such, they cannot recapitulate the chronicity of a multifactorial disease as complex as NAFLD (Chavez-Tapia, Rosso, & Tiribelli, 2012; Gómez-Lechón et al., 2007a; Gori et al., 2014; Ricchi et al., 2009). In this regard, the emerging organ-on-chip technology can be the real game changer: these platforms represent dynamic cell culture systems that offer great promise for simulating and studying human diseases (Liu et al., 2019; Rothbauer et al., 2019), including NAFLD, at tissue and organ level (Bovard et al., 2018; Chang et al., 2017; Li, George, Vernetti, Gough, & Taylor, 2018; Lu et al., 2018; "Organ network in transparent chip to study how cancer cells spread," n.d.). Recently, our group[4] and others (Bulutoglu et al., 2019; Ehrlich et al., 2018; Lee & Sung, 2018) have proposed alternative and more complex models of hepatic steatosis in a microfluidic chip to overcome the bottlenecks and limitations of the canonical *in vitro* cell culture models.

Furthermore, organs-on-chips are being adapted to high-throughput screening for drug discovery, also in combination with high-content imaging systems (Lin, Ballinger, & Khetani, 2015; Peel et al., 2019; Tan et al., 2019; van den Berg, Mummery, Passier, & van der Meer, 2019).

Thus, leveraging this approach/technology, here we disclose an advanced three-dimensional (3D) culture model of NAFLD (using perfused HepG2 cells) within a liver-on-a-chip microfluidic device, under dynamic culture conditions. This platform, by mimicking the endothelial-parenchymal interface of a liver sinusoid in an optimized 3D cell culture microenvironment, allowed us to investigate the pathogenesis of NAFLD, through the high-content analysis (HCA) of different parameters of liver steatosis at the cell level. Finally, the NAFLD-on-a-chip model has been exploited to study the protective effects of the two aforementioned naturally occurring plant-derived polyphenols, namely Quercetin and Hydroxytyrosol, on the development of NAFLD as potential therapeutic treatment.

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Euroclone (MI, Italy); L-glutamine, penicillin-streptomycin mixture, and AdipoRed Assay Reagent were purchased from Lonza (East Rutherford, NJ, USA); MEM non-essential amino acid solution M7145, palmitic acid (PA; C16:0), oleic acid (OA; C18:1 cis-9), bovine serum albumin (BSA) Cohn fraction V, Hydroxytyrosol (HT) and Quercetin (Que) were purchased from Merck (Darmstadt, Germany); Fetal Bovine Serum (FBS, Gibco), charcoal-stripped FBS, Hoechst 33342 fluorescent nuclear dye and ethidium homodimer-1 (EthD-1) were purchased from Thermo Fisher Scientific (Waltham, MA, USA); ROS-ID Total ROS/Superoxide Detection Kit (ENZ-51010) was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA).

Microfabrication

The geometry of the microfluidic device was designed using a CAD suite (Layout Editor, Juspertor UG, Unterhaching, Germany) and fabricated in polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland,

MI), based on the previously published model (Gori et al., 2016), through the same two-layer soft-lithographic process to pattern the cell culture microchamber together with the transport channels and the endothelial-like barrier, respectively.

Cell culture and chip loading

Cell culture. The human hepatoma cell line HepG2/C3A (CRL-10741) was purchased from the American Type Culture Collection (ATCC). Cells were expanded in DMEM low glucose supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, MEM non-essential amino acid solution (100x), 10% FBS, and incubated in a humidified 37 °C atmosphere containing 5% CO_2.

Chip loading. HepG2 cells were detached at subconfluence, and loaded into chips at a density of 1.0×10^6 cells/mL*via* the central cell loading channel as previously described (Gori et al., 2016). Thereafter, cells were cultured on chip, in 3D fashion, under microfluidic perfusion obtained by applying a height differential to the culture medium contained in the inlet/outlet reservoirs. The sinusoid-like microarchitecture of the chip allowed the continuous diffusion of nutrients and the removal of metabolic waste products, with negligible shear stress thanks to the microchannel barrier. After chip loading, cells were cultured overnight (o.n.) in standard culture medium; the next day, medium was discarded and replaced with fresh steatosis medium containing FFAs and polyphenols according to the different treatments as described in the following.

Cell treatments

Induction of steatosis. For the induction of hepatic steatosis, a combination of long-chain FFAs, namely PA and OA was dissolved in methanol (vehicle). Each of the two FFAs was added to the medium, either alone or in combination at different molar ratios (i.e., 1:2 and 2:1, respectively), at 1mM final concentration, which is in the range concentration of FFAs in human plasma (i.e. 0.2-2mM) (Feldstein et al., 2004) for 48 hours (h). PA and OA were chosen as they are the most abundant FFAs in western diets and components of liver triglycerides in both normal subjects and patients with NAFLD (Baylin, Kabagambe, Siles, & Campos, 2002; Gómez-Lechón et al., 2007b). Steatosis was induced by modifying the previously described method (Gori et al., 2014, 2016). Briefly, HepG2 cells were incubated in steatosis medium composed of DMEM (low glucose) supplemented with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, 1% BSA, 10% charcoal-stripped FBS, and 1 mM FFAs at the above described ratios. Internal controls were represented by steatosis medium without FFAs but with equivalent volume of methanol vehicle. The effects of the FFA treatments in terms of intracellular lipid accumulation, cell viability and oxidative stress were evaluated after 48h in culture *via* confocal microscopy-based high-content screening (HCS), using fluorescence-based functional assays.

Cell treatments with polyphenols. QUE and HXT were both used at a physiological concentration of 10 µM (Catalán et al., 2015; Hur et al., 2012; Radtke, Linseisen, & Wolfram, 2002; Vidyashankar et al., 2013) for 48h, in the steatosis medium, separately or with the diverse combinations of FFAs.

Evaluation of hepatic steatosis

The intracellular accumulation of TGs was measured by the AdipoRed assay

according to the manufacturer's instructions. Briefly, after cell treatments for 48h, chips were rinsed with PBS and incubated with AdipoRed reagent in PBS at room temperature (RT) for 1h, and mean fluorescence intensity (MFI) of the regions of interest (ROIs) occupied by the cells was measured using a laser scanning confocal microscope (A1R MP on an Eclipse Ti-2 inverted microscope, Nikon, Tokyo, Japan) with automated acquisition/analysis software (NIS Elements AR, Nikon), and plotted as fold increase compared to the control (vehicle only).

Analysis of cell viability/cytotoxicity

After cell treatments for 48h, chips were rinsed with PBS and incubated at RT for 1h with blue-fluorescent nuclear dye Hoechst 33342 (5 µg/mL in PBS) that stains all nuclei, and the red-fluorescent dye EthD-1 (4 µM in PBS) that is selective to nuclei of dead cells. The number of dead*vs.* total cells laying in each cell

culture microchamber was visualized under a laser scanning confocal microscope (Nikon A1R) and counted through an automated measurement routine (NIS Elements AR). Results were plotted as a percentage of live cells in FFAs- and polyphenols- treated *vs.* control cultures.

Measurement of cell ROS/Superoxide production

For the evaluation of oxidative stress in live cells, the ROS-ID Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Inc), was used according to the manufacturer's instructions. Briefly, after cell treatments for 48h, cells were incubated with reactive oxygen/nitrogen (ROS/RNS) detection reagent and Superoxide detection reagent both used at a final concentration of 3 μM in wash buffer for 1h in the dark in a humidified 37 °C incubator. After incubation, chips were washed with wash buffer and immediately observed. For positive controls, chips were simultaneously incubated with the ROS Inducer (Pyocyanin) in wash buffer at 500 μM, and with the detection probes for 30 min. at 37 °C in the dark. Chips were observed under a confocal microscope (A1R MP Eclipse Ti-2, Nikon) and MFIs of the ROIs occupied by the cells were measured *via* an automated acquisition/analysis software (NIS Elements AR, Nikon). ROS/RNS were detected using a 488 nm laser line with a FITC filter set (525/50 nm band-pass, BP), while Superoxides were detected using a 561 nm laser line with a TRITC filter set (595/50 BP). MFI levels were subtracted for endogenous fluorescence of untreated cells. Results were plotted as the ratio between each treatment and its own internal control.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM) of at least three independent experiments. Data were analyzed using GraphPad Prism 7.04 software. Statistical significance was assessed by one-way analysis of variance (ANOVA), followed by Tukey post hoc testing. Significance was at the 0.05 level.

Results

Development of the microfluidic model of hepatic steatosis

In the present work, we first developed an in vitro model of hepatic steatosis, under 3D dynamic culture conditions, into a microfluidic sinusoid-like device (Liver-on-a-Chip device in Fig. 1) based on the model previously published (Gori et al., 2016). Hepatic steatosis was induced in HepG2 cells providing a fat overload with mixtures of two FFAs (PA and OA), added to the medium alone or in combination at 1:2 and 2:1 molar ratios, and at 1mM final concentration for 48h. Depending on the different proportions of saturated and unsaturated fatty acids used, may represent hepatic cellular models of steatosis that mimic benign chronic steatosis or a more severe and acute condition of steatosis [6]. Then, through the use of confocal microscopy-based HCA we evaluated in real-time the following parameters of steatosis: intrahepatic FFA accumulation in the form of TGs (Fig. 2a, b) and their cytotoxicity (Fig. 3a, b) as well as global levels of ROS/RNS and Superoxide species (Fig. 4a-c) generated as a result of the different FFA overloads.

[Figure 1]

Analysis of intrahepatic triglyceride accumulation, cytotoxicity and oxidative stress in our microfluidic model of NAFLD

After the treatments with the different mixtures of FFAs for 48h (Fig. 2a, and b in the representative confocal micrographs), only an overload of 1mM OA produced a statistically significant intracellular accumulation of triglycerides [a four-fold increase *vs.* control (ctrl), $p<0.05$]. The intracellular storage of triglycerides observed in the other treatments, was proportional to the prevalence of OA compared to PA. The analysis of FFA-induced cytotoxicity (Fig. 3a, and b in the representative confocal micrographs) showed a remarkable decrease in cell viability with 1mM PA (approximately 30% reduction *vs.* control, $p<0.0001$), followed by the treatments with PA:OA 2:1 ($p<0.01$) and PA:OA 1:2 ($p<0.01$), implying a clear dependence on the amount of PA in the FFA mixture. On the contrary, the 1mM administration of OA alone did not cause any significant reduction in cell viability compared to the ctrl. Lastly, the evaluation of the oxidative stress derived from exogenous FFA overload showed a statistically significant increase of both total ROS/RNS levels only in the PA and PA:OA 2:1 treatments (Fig. 4a and c in the representative confocal micrographs), and Superoxide

levels only in the PA group (Fig. 4b and c in the representative confocal micrographs) compared to ctrl. Conversely, no significant rise was detected in any of the other treatments, thereby underlining a direct effect of PA in the generation of oxidative stress.

[Figure 2]

Overall, it turned out that while OA is more steatogenic than PA (Fig. 2a, b), the latter is more cytotoxic than OA (Fig. 3a, b), which is consistent with the literature (Gómez-Lechón et al., 2007b; Ricchi et al., 2009). Finally, in agreement with previous works (Gori et al., 2016; Ricchi et al., 2009), the overload of FFAs produced moderate levels of total ROS/RNS and Superoxide species (Fig. 4a-c) in every treatment except those with PA and PA:OA 2:1, which showed higher increases most likely responsible for the cytotoxic effects observed in Fig. 3 and that may then lead to inflammation and apoptosis (Assaily et al., 2011; Gori et al., 2014) correlated to disease progression and severity (Angulo, 2002; Feldstein, Canbay, Angulo, et al., 2003; Feldstein, Canbay, Guicciardi, et al., 2003).

[Figure 3]

Quercetin and Hydroxytyrosol individually reduce intrahepatic triglyceride accumulation induced by the FFAs

Next, we investigated the role of dietary concentrations of the two polyphenols in FFA-induced hepatic steatosis: i) Quercetin, a flavonoid widely distributed in nature in many foods, especially in vegetables, fruits and tea, at 10 μM final concentration, which is in the average circulating human plasma concentrations, and in line with previous reports (Radtke et al., 2002; Vidyashankar et al., 2013); ii) Hydroxytyrosol, produced by the hydrolysis of oleuropein, which is a polyphenol peculiar to olives and olive oil (Hur et al., 2012), at physiological concentration of 10 μM (Hur et al., 2012). These two natural compounds have been selected for their antioxidant, anti-inflammatory and hypolipidemic properties (Hur et al., 2012; Marcolin et al., 2012; Park, Choi, Um, Yoon, & Park, 2011; Peres et al., 2000; Priore et al., 2014; Vidyashankar et al., 2013) and, in turn, for the possibility to restrain or prevent the development of NAFLD in our microfluidic model (i.e., reducing lipid accumulation, and the related lipotoxicity and oxidative stress). Interestingly, when 10 μM Quercetin (Fig. 2c, and 2d in the representative confocal micrographs) and 10 μM Hydroxytyrosol (Fig. 2e, and 2f in the representative confocal micrographs) were separately added for 48h to the cells, along with the different mixtures of FFAs, intrahepatic triglyceride accumulation was remarkably reduced compared to FFAs alone (Fig. 2a, b), with a statistically significant difference in the OA treatment (Fig. 2c, e, $p<0.05$ and $p<0.01$ for Que and HT, respectively) that is the most steatogenic. Hence, such hypolipidemic effect of Que and HT showed a very similar trend in all treatments and thus a relatively comparable effect in the context of hepatic steatosis, which was further confirmed by the log2-fold change (log2 F.C. in Fig. 1Sa and b) analysis of the AdipoRed experiment. Indeed, the present analysis showed that the more the prevalence of OA in the FFA mix and the stronger the lipid-lowering effect of both Que and HT with the latter that, regardless of the treatment, presents more statistically significant fold changes compared to Que.

Quercetin and Hydroxytyrosol protect from the lipotoxicity of FFAs

Furthermore, a corresponding significant decrease in cytotoxicity was also observed in almost all the treatments with Quercetin (Fig. 3c and representative confocal micrographs in 3d) and Hydroxytyrosol (Fig. 3e and representative confocal micrographs in 3f) compared to the FFAs alone. Notably, regarding the strong lipotoxicity of PA, its effect was partially reduced by both polyphenols compared to the treatments w/o polyphenols, except for the PA:OA 1:2 condition in which the cell viability increase, both after Que and HT administration, became statistically significant (as shown in Fig. 3c, $p<0.01$, for Que, and in Fig. 3e, $p<0.01$, for HT). Nevertheless, when the combined treatments of PA with Que (Fig. 3c) and HT (Fig. 3e) were compared to that with PA alone (white bars), we detected a slight increase in cell viability that, however, was not statistically significant in either case.

Quercetin and Hydroxytyrosol lower oxidative stress generated by the FFAs

Finally, our data showed that also total ROS/RNS and Superoxide levels were dramatically lowered with the

addition of Quercetin (Fig. 4d, e and f in the representative confocal micrographs) and Hydroxytyrosol (Fig. 4g, h and i in the representative confocal micrographs) compared to the FFAs alone (Fig. 4a, b and c in the representative confocal micrographs), as also proved by the log2-fold change analysis for both polyphenols in the (log2 F.C. in Fig. 1Sc-f). In particular, the most powerful effect of both polyphenolic compounds was observed against the PA-induced ROS/RNS and Superoxide species in which the downregulation was statistically significant ($p<0.01$ in Fig. 4d and e for Que, and $p<0.05$ in Fig. 4g and h for HT). In addition, in the PA:OA 2:1 treatment, the ROS/RNS and Superoxide production was significantly lowered by both Que and HT (Fig. 4d and e with $p<0.001$ for Que; Fig. 4g and h with $p<0.01$ for HT, respectively), highlighting a slightly stronger effect, in terms of statistical significance, of Quercetin against oxidative stress compared to Hydroxytyrosol at the chosen concentrations.

[Figure 4]

Discussion

Drug discovery is currently hindered by the inability of conventional 2D cell culture models as well as animal experiments to accurately predict human responses. Liver-on-a-chip platforms may revolutionize this scenario by reproducing the natural 3D microenvironment of the cells and recapitulating some functionality of the hepatic tissue, allowing us to imitate liver pathophysiology more closely to its *in vivo*counterpart. Hence, we leveraged on such disease-on-a-chip technology to model the condition of hepatic steatosis, and to investigate at the cell level the protective effects of dietary concentrations of two natural polyphenols against some important features of the disease, in particular the intrahepatic fat accumulation and its related lipotoxicity and oxidative stress. Regarding Quercetin, its therapeutic potential and hepatoprotective effect has been thus far attributed to its antioxidant, anti-inflammatory and hypolipidemic activity (Marcolin et al., 2012; Peres et al., 2000; Vidyashankar et al., 2013). Instead, the beneficial effect in human health of extra virgin olive oil has been long ascribed to its high content of oleic acid (Carluccio, Massaro, Scoditti, & De Caterina, 2007; María-Isabel Covas, Konstantinidou, & Fitó, 2009; Esposito & Giugliano, 2010). However, more recently, also the important role played by the phenolic components (such as the oleuropein-derivative Hydroxytyrosol) has been increasingly emerging, not only for their known anti-oxidant and anti-inflammatory properties, but also for their lipid-lowering ability (Bendini et al., 2007; Carluccio et al., 2003; M.-I. Covas, 2008; Gordon, Paiva-Martins, & Almeida, 2001; Hur et al., 2012; Jemai, Fki, et al., 2008; Jemai, Bouaziz, Fki, El Feki, & Sayadi, 2008; Park et al., 2011; Pérez-Jiménez, Ruano, Perez-Martinez, Lopez-Segura, & Lopez-Miranda, 2007; Priore et al., 2014). Indeed, in this work we observed a stronger effect of HT, compared to Que, in mitigating the steatogenic effect of OA as well as the different mixtures of FFAs (see Fig. 2 and Fig. 1S). Actually, our *in vitro* results on the effects of Que and HT in the framework of NAFLD confirmed *in vitro* as well as *in vivo* data from the literature (Hur et al., 2012; LI et al., 2013; Pirozzi et al., 2016; Porras et al., 2017; Priore et al., 2014; Valenzuela et al., 2017; Vidyashankar et al., 2013) but in a more realistic scenario that is closer to the *in vivo* situation. In fact, our microfluidic model of NAFLD, providing us a more physiological setting than conventional static 2D culture systems, may represent a more suitable platform for simulating the chronicity of the disease and, as such, getting closer to the animal model, despite its intrinsic limitations that have yet to be completely overcome and improved.

These findings very importantly show a protective and comparable effect of Quercetin and Hydroxytyrosol against FFA-induced hepatic steatosis (confirming their lipid-lowering activity), lipotoxicity and oxidative stress, in which they are able to scavenge free radicals. Therefore, their role in counteracting excessive ROS/Superoxide generation and boosting the antioxidant defenses of hepatic cells, along with the reduction of excessive fat accumulation, seems to promote cell viability, and may represent an appealing strategy for the treatment of NAFLD. To date, no regulatory agency- approved cure for NAFLD has been found yet. As a matter of fact, the results reported herein together with the many beneficial pharmacological effects of Quercetin and Hydroxytyrosol on liver damage may promote their future clinical application as safe and effective therapeutic agents (Cao et al., 2014; Echeverría et al., 2018; Marcolin et al., 2012; Peres et al., 2000; Pirozzi et al., 2016; Tang et al., 2016; Valenzuela et al., 2017). Future experiments will also include: i) the combined administration of the two polyphenols to steatotic cells, in order to investigate a possible

synergistic effect of the two compounds; ii) the use of a more complex liver microarchitecture that will involve different cell types, including other parenchymal (i.e., primary human hepatocytes or iPSC-derived hepatocytes) and non-parenchymal liver cells (e.g., endothelial cells, Kupffer cells and hepatic stellate cells) to enable also the analysis of the expression levels of inflammatory and fibrogenic cytokines (e.g., IL-6, IL-8, IL-1β, TNF-α, TGF-β1, CTGF), involved in the development of NAFLD, from the supernatants of such a multicellular hepatic microenvironment. In conclusion, our NAFLD-on-a-chip approach may also pave the way to the technological advancement of drug research, providing a promising tool to face the challenges of drug screening with the final goal, in the next future, to connect different tissues or even organs into a complex model system for the study of human development and disease.

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following Quercetin and Hydroxytyrosol addition to the different FFA mixtures. The fold decrease in intracellular triglyceride accumulation (*via* the AdipoRed assay) due to Que (a) and HT (b) administration is reported; the fold decrease in oxidative stress (ROS/RNS in c and d, and Superoxide species in e and f, respectively) due to Que (c and e) and HT (d and f) administration is reported. All values are reported as mean ± SEM, n = 3 at least, and plotted as the log2 of the ratio between the different mixtures of FFAs w/ Que or HT and the FFAs alone normalized *vs.* blank control (i.e., no effect); statistical significance was evaluated by one-way ANOVA, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$. Legend: Ctrl (Control); Que (Quercetin); HT (Hydroxytyrosol).