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PPAR agonists reduce steatosis in oleic acid-overloaded HepaRG cells

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List of Abbreviations

FAO: fatty acid oxidation; MTT: methylthiazoletetrazolium; NAFLD: non-alcoholic fatty liver diseases; NASH: non-alcoholic steatohepatitis; OA: oleic acid; PPAR: Peroxisome proliferator-activated receptor

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ABSTRACT

Although non-alcoholic fatty liver disease (NAFLD) is currently the most common form of chronic liver disease there is no pharmacological agent approved for its treatment. Since peroxisome proliferator-activated receptors (PPARs) are closely associated with hepatic lipid metabolism, they seem to play important roles in NAFLD. However, the effects of PPAR agonists on steatosis that is a common pathology associated with NAFLD, remain largely controversial. In this study, the effects of various PPAR agonists, *i.e.* fenofibrate, bezafibrate, troglitazone, rosiglitazone, muraglitazar and tesaglitazar on oleic acid-induced steatotic HepaRG cells were investigated after a single 24-hour or 2-week repeat treatment. Lipid vesicles stained by Oil-Red O and triglycerides accumulation caused by oleic acid overload, were decreased, by up to 50%, while fatty acid oxidation was induced after 2-week co-treatment with PPAR agonists. The greatest effects on reduction of steatosis were obtained with the dual PPARα/γ agonist muraglitazar. Such improvement of steatosis was associated with up-regulation of genes related to fatty acid oxidation activity and down-regulation of many genes involved in lipogenesis. Moreover, modulation of expression of some nuclear receptor genes, such as FXR, LXRα and CAR, which are potent actors in the control of lipogenesis, was observed and might explain repression of *de novo* lipogenesis. **Conclusion:** Altogether, our *in vitro* data on steatotic HepaRG cells treated with PPAR agonists correlated well with clinical investigations, bringing a proof of concept that drug-induced reversal of steatosis in human can be evaluated in *in vitro* before conducting long-term and costly *in vivo* studies in animals and patients.

Key words: hepatotoxicity; non-alcoholic steatohepatitis; fatty acid oxidation; fatty acid overload; hepatocytes

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is currently the most common form of chronic liver disease and is strongly associated with obesity, type 2 diabetes and insulin resistance (Caldwell and Argo, 2010 ; Lomonaco *et al.,* 2013). Considered as the hepatic manifestation of the metabolic syndrome, NAFLD encompasses a wide spectrum of clinical-histological disturbances ranging from simple triglycerides accumulation in hepatocytes (hepatic steatosis) to hepatic steatosis with inflammation [non-alcoholic steatohepatitis (NASH)], fibrosis, cirrhosis and in severe cases, hepatocarcinoma.

The pathogenesis of fatty liver is intimately related to derangements in nutrient metabolism and energy homeostasis, which are tightly regulated by endocrine, paracrine, and autocrine signals that control the expression and activity of key metabolic enzymes by transcriptional and post-transcriptional mechanisms. Peroxisome proliferator-activated receptors (PPARs) have recently emerged as a master class of ligand-activated transcription factors involved in maintaining lipid homeostasis and energy balance, as well as a variety of other biological processes. PPARs comprise three subtypes: PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3), that each mediates distinct physiological effects on lipid metabolism and glucose homeostasis (Michalik *et al.*, 2006). PPARα plays a pivotal role in fatty acid catabolism in liver by transcriptionally up-regulating the expression of some of the key genes involved in fatty acid oxidation (FAO) (Reddy and Hashimoto, 2001). PPARβ/δ is a powerful metabolic regulator expressed ubiquitously that also exerts key functions in liver by enhancing fatty acid catabolism (Barish *et al.*, 2006). PPARγ is mainly expressed in adipose tissue and is known to be a regulator of lipid metabolism and glucose homeostasis.

Synthetic PPAR agonists represent attractive drug targets for the management of NAFLD and NASH. PPARα activators, typified by fibrates, are used to treat dyslipidemia while PPARγ ligands, such as thiazolidinediones, act as insulin sensitizers in type 2 diabetes mellitus. Both agonist classes have been reported to attenuate NASH. Thus, fibrates, such as fenofibrate, have been shown to reduce and/or prevent fatty liver in various experimental models (Nagai *et al.*, 2002; Ye *et al.*, 2002). Their effects have been attributed to an increase in hepatic FAO activity, however some discrepancies have been observed in human NASH treated with fibrates. A 12-month treatment of patients with NASH and elevated triglycerides with

clofibrate had no impact on liver enzyme elevation or triglyceride levels (Laurin *et al.*, 1996). In fact, the human liver expresses relatively low levels of PPARα and PPARα activation, and has little impact on FAO in contrast to its rodent counterpart (Kane *et al.*, 2006). The PPAR pan-agonist, bezafibrate, which modulates FAO by influencing both PPARα and PPARδ, was also found to reduce hepatic steatosis (Nagasawa *et al.*, 2006) and improve NASH (Nagasawa *et al.*, 2006; Lim *et al.*, 2009).

The effects of PPARγ agonists on steatosis and NAFLD are also controversial. While PPARγ expression and activity are low in normal liver they are usually increased in steatotic liver of both animals (Kim *et al.*, 1998) and humans (Gavrilova *et al.*, 2003). Nevertheless, although PPARγ is considered as a steatogenic factor, its activation is a putative target for the treatment of NAFLD. Indeed, thiazolidinediones (e.g. pioglitazone and rosiglitazone) and metformin are the principal insulin sensitizing agents employed in the management of NAFLD for their capacity to reduce amino-transferase levels and to improve histological grading (Balas *et al.*, 2007), probably as a consequence of their primary insulin-sensitizing effects on adipose tissue (Tanaka *et al.*, 2003).
Dual PPARα/γ agonists have also been considered to be attractive as therapy for NAFLD, NASH and the metabolic syndrome as they have been reported to have the potential to improve insulin sensitivity and decrease circulating triglycerides in animals. However, the first molecules, muraglitazar and tesaglitazar, have been withdrawn from phase III clinical trials due to increased incidence of cardiac and renal toxicities (Hamren *et al.*, 2008; Rubin *et al.*, 2009).

With the global prevalence of metabolic syndrome, obesity and NAFLD in recent years, there is an increasing need to better understand the role of PPAR agonists on the etiology of fatty liver disease. The aim of this study was to analyze the effects of the different types of PPAR agonists (glitazones, glitazars and fibrates) on steatosis induced by oleic acid overload in differentiated human HepaRG cells after acute and repeat treatments (Antherieu *et al.,* 2011). A decrease in triglycerides content associated with an increase in FAO-related gene expression and activity was observed with all agonists after 14-day repeat exposure. The strongest effects were obtained after treatment with the dual PPARα/γ agonist muraglitazar.

MATERIAL AND METHODS

Chemicals. Troglitazone, rosiglitazone, muraglitazar and tesaglitazar were provided by the Servier Chemical Department. Fenofibrate, bezafibrate and oleic acid (OA) were purchased from Sigma (St. Quentin Fallavier, France). [U-^{14}C]-palmitic acid was from Perkin Elmer (Boston, MA).

HepaRG cells. The untransformed human HepaRG cell line is derived from a human cholangiohepatocarcinoma. The cells used in this study were from cells stored frozen at passage 10 and experiments were performed between passages 13 and 18. Briefly, HepaRG cells were usually seeded at a density of 2.6 x10^4 cells/cm^2 in the Williams' E medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL insulin, 2 mM glutamine and 50 µM hydrocortisone hemisuccinate (Gripon *et al.*, 2002). After two weeks, HepaRG cells were transferred to the same medium supplemented with 2% dimethylsulfoxide (DMSO) for further two weeks in order to obtain confluent differentiated cultures containing both hepatocyte-like and progenitors/primitive biliary-like cells (around 50% of each type) (Cerec *et al.*, 2007). In the presence of DMSO the levels of certain functions, in particular those of some major cytochromes P450 such as CYP3A4, are markedly increased (Aninat *et al.*, 2006; Kanebratt and Andersson, 2008; Antherieu *et al.*, 2010).

Chemical treatments. The PPAR agonists and OA were dissolved manually in dimethylsulfoxide. HepaRG cell cultures were first exposed to 250 µM OA for 24 hours, then continuously to OA either alone or together with each PPAR agonist for 24 hours or repeated treatments every 2-3 days for 14 days (Figure 1). DMSO dilutions were made from a 100% DMSO solution. At least three independent experiments were performed in triplicates.

Cell viability. Cytotoxicity was evaluated using the methylthiazoletetrazolium (MTT) colorimetric assay. After treatment, medium was removed and serum-free medium containing 0.5 mg/mL MTT was added to each well and incubated for 2 hours at 37°C. After removal of the incubation solution, water-insoluble formazan was dissolved in DMSO and absorbance was measured at 550 nm.

Oil Red O staining and lipid droplets analysis by cell imaging. Neutral lipid accumulation was determined by Oil Red O staining, which allows detection of triglycerides and cholesterol esters. A stock solution of Oil Red O was prepared in isopropanol (0.5:100). After each

treatment, cells were incubated for one hour with Oil Red O-saturated solution (isopropanol:water, 3:2) and then observed under phase-contrast microscopy. To quantify lipid accumulation, Oil Red O-stained lipid droplets were analyzed by cell imaging. Areas of stained droplets were determined by the Image J software and normalized by the areas of hepatocyte clusters.

Triglycerides quantification. Triglycerides were extracted using an organic solvent chloroform/methanol (2/1 v/v). A serum triglycerides determination kit (Sigma) was used for quantification, using a spectrophotometer with absorbance at 550 nm according to the manufacturer's instructions.

Real time-quantitative polymerase chain reaction analysis (RT-qPCR). Total RNA was extracted from 10^6 HepaRG cells using the SV total RNA isolation system (Promega). RNAs were reverse-transcribed into cDNA and RT-qPCR was performed using a SYBR Green mix and a Step One plus equipment (Applied Biosystems). Primer sequences are listed in Table 1.

Fatty acid oxidation. HepaRG cells were incubated with a medium containing 0.5 mM L-carnitine, 10% fat-free bovine serum albumin and [U-^{14}C]-palmitic acid (final concentration, 1 mM; 0.05 µCi/mL). The reaction was carried out for 90 min at 37°C. After addition of perchloric acid (final concentration, 3%) and centrifugation (4000*g*; 10 min) an aliquot of the supernatant was sampled and counted for [^{14}C]-labelled acid-soluble β-oxidation products (Fisch *et al.*, 1996).

Statistical analysis. Each value corresponded to the mean ± standard error of mean (S.E.M) of three independent experiments in triplicates. The Mann-Whitney *U* test was applied to compare data between OA- or drug-treated cell cultures and their control counterparts. Data were considered significantly different when $p<0.05$.

RESULTS

Cytotoxic effects of PPAR agonists in steatotic HepaRG cells

Preliminary experiments were performed to assess cytotoxicity of the different PPAR agonists in steatotic HepaRG cells over a 14-day period. In order to induce vesicular steatosis, HepaRG cells were exposed to 250 µM OA for 24 hours (Antherieu *et al.*, 2011) prior to co-treatment with PPAR agonists. Noticeably, when OA-overloaded HepaRG cells were maintained in culture without repeat exposure to the fatty acid, vesicular steatosis disappeared within 5 days; consequently effects of PPAR agonists were investigated with simultaneous addition of OA during the 14-day treatment.

Neither cellular morphological alterations nor loss of cell viability were observed in untreated cultures over a 14- day period. OA alone did not cause any cytotoxicity after 1-day exposure while after a 14-day treatment slight toxicity was observed not exceeding a 12% decrease in MTT values. All compounds were added to the cultures at a high concentration corresponding to IC10, *i.e.* the concentration that caused 10% loss of cell viability in OA-overloaded HepaRG cells after 14-day treatment (Figure 2). Accordingly, examination of the cultures under phase-contrast microscopy did not reveal marked cellular morphological alterations after a 14-day treatment whatever the PPAR agonist tested. Consequently, 25 µM fenofibrate and bezafibrate, 40 µM troglitazone, 50 µM rosiglitazone and muraglitazar and 300 µM tesaglitazar were used for further experiments.

Oil Red O staining and quantification of lipid droplets by cell imaging

Cells were first exposed to OA for 24h and then co-treated with troglitazone, rosiglitazone, muraglitazar, tesaglitazar, fenofibrate and bezafibrate for either 1 or 14 days and neutral lipids (triglycerides and cholesterol esters) were stained by Oil Red O. Cells were examined under phase-contrast microscopy and photographed (Figure 3A) before quantification of intracellular Oil Red O accumulation by cell imaging (Figure 3B). As previously reported (Antherieu *et al.,* 2011), numerous vesicles stained with Oil Red O were observed in the cytoplasm of HepaRG hepatocytes after 1- and 14-day incubation with OA under light microscopy. After 1-day co-treatment with PPAR agonists, intracellular distribution of lipid vesicles was not affected while their intracytoplasmic surface appeared to be reduced by about

50% after 14 days with rosiglitazone, muraglitazar, tesaglitazar, fenofibrate and bezafibrate. By contrast, troglitazone did not cause detectable variations in intracytoplasmic lipid staining when compared with cultures incubated with OA alone.

Triglycerides quantification

Triglycerides levels were determined by spectrophotometry (Figure 4). OA induced a 2-fold increase in triglycerides after 1 day and no further change was observed over a 14-day period. Co-treatment with PPAR agonists for 1 day did not show significant difference compared to untreated OA-overloaded cells. After repeat exposure, a slight decrease in triglycerides content was observed with troglitazone whereas more pronounced reduction was demonstrated with the other PPAR agonists, especially rosiglitazone and muraglitazar. Indeed, rosiglitazone and muraglitazar decreased triglycerides levels to approximately 50 and 60 % of the values measured in the OA-treated cultures respectively.

Induction of fatty acid oxidation

Since FAO was described as an important pathway involved in the modulation of steatosis, the effects of PPAR agonists on FAO activity were also assessed (Figure 5). Cells were incubated with [^{14}C]-palmitate and FAO was estimated by measuring [^{14}C]-labelled acid-soluble ß-oxidation products. OA overload did not significantly modulate FAO activity whatever the treatment duration. No effect of PPAR agonists was also noticed after 1-day treatment. By contrast, FAO activity was increased after 14-day repeat treatment with all PPAR agonists. The highest increases were obtained with muraglitazar, bezafibrate and rosiglitazone, reaching 1.7-fold with muraglitazar relative to untreated control. In addition, a dose-response analysis of the effects of rosiglitazone and muraglitazar on fatty acid oxidation activity was performed and demonstrated that the highest effects were obtained with the highest concentration of each agonist (data not shown).

mRNA expression modulation of key genes involved in lipid metabolism

In order to better investigate the mechanisms involved in steatosis reduction in OA-overloaded HepaRG cells, changes in the expression of several genes, which are key players in lipid metabolism were examined by RT-qPCR after 1- and 14-day treatments with PPAR

agonists (Table 2). These genes are involved in *de novo* lipogenesis (ELOVL6, FASN, PPARG, SCD1, SREBP1 and THRSP), FAO and mitochondrial biogenesis (ACADL, ACOX1, CPT1A, CPT2, ECH1, CYP4A11, HADHA and PPARA), lipid hydrolysis and formation of lipid droplets (ADFP, PLIN4 and LPL), lipoprotein synthesis (APOC3), nuclear receptors (LXR, FXR, PXR and CAR) as well as other liver functions (ALB and CYP2B6).

As previously reported, CTP1A, ADFP and PLIN4 were overexpressed and THRSP was repressed after 1-day and 14-day OA-overload (Antherieu *et al.,*2011). Almost all genes were modulated by one or more PPAR agonists at least at one time-point after 1- and 14-day co-treatments with OA. A noticeable up-regulation of the genes related to FAO, mitochondrial biogenesis, lipid hydrolysis and formation of lipid droplets was observed after 1-day treatment, with the highest fold changes after muraglitazar exposure, especially for ACOX1, CPT1A, CPT2, ECH1, ADFP and PLIN4. After 14-day treatment, expression of these genes remained most important for muraglitazar and rosiglitazone. Deregulated genes related to *de novo* lipogenesis, except THRSP, were also overexpressed after 1 day whereas they were mostly unchanged or even decreased after 14 days, for each PPAR agonist. It is noteworthy that APOC3, the main endogenous inhibitor of LPL, was overexpressed by rosiglitazone and the two glitazars after 14-day treatment.

To understand how modulation of related FAO and *de novo* lipogenesis genes might occur, expression of some nuclear receptors, such as LXR, FXR, PXR and CAR, were also investigated. After 1 day, LXRα, the main inducer of SREBP1, was overexpressed by all the test compounds, especially the two glitazones. By contrast, after 14 days, LXRα expression was dramatically decreased by fenofibrate, troglitazone and tesaglitazar. The second isoform LXRβ did not show any significant change after either 1- or 14-day treatments (data not shown). The nuclear receptor FXR was also up-regulated after 1 day by bezafibrate and muraglitazar and it was still significantly overexpressed after 14 days by muraglitazar only.

PXR and CAR mRNA levels were similarly measured, because of their potential role in the regulation of lipid metabolism. After 1-day or 14 days treatments PXR expression was either unchanged or slightly down- or up-regulated. CAR expression was up-regulated after 1 day by both glitazones, fibrates, and muraglitazar whereas after 14 days, it was still deregulated only by muraglitazar, with a 2.2-fold increase relative to untreated control.

ALB and CYP2B6 genes were also found to be modulated. After a 14-day treatment albumin transcripts were augmented with both glitazones and glitazars while they were decreased by

fenofibrate and bezafibrate. As expected (Rogue *et al.,*2011), CYP2B6 was specifically overexpressed by the two glitazones at both time-points.

DISCUSSION

Although NAFLD is currently the most common form of chronic liver disease there is currently no marketed drug for its treatment (Lomonaco *et al.*, 2013). As PPAR agonists influence hepatic lipid homeostasis and energy balance, they represent an attractive target for the development of novel drug therapies in the management of NAFLD. However, although activation of the three classes of PPARs by synthetic ligands has been shown to reduce hepatic steatosis in rodents, discrepancies still remain with data obtained from clinical studies in humans (Laurin *et al.*, 1996; Mahady *et al.*, 2011; Lomonaco *et al.*, 2013). In the current work, we showed that repeat activation of PPARs by several agonists resulted in reduction of steatosis using fatty acid-overload human HepaRG cells (Antherieu *et al.*, 2011). Improvement of OA-induced steatosis was evidenced by a decrease in intracellular lipid droplets and triglycerides content, induction of FAO and overexpression of genes related to FAO and mitochondrial biogenesis after a 2-week treatment. The most potent effects were obtained with the dual PPAR agonist muraglitazar and to a lesser extent with rosiglitazone. The choice of PPAR agonists concentrations was based on IC10 values determined by the MTT test in 14-day cultures; such values are frequently used for functional studies *in vitro*. Thus, 25 µM fenofibrate, 25 µM bezafibrate, 40 µM troglitazone, 50 µM rosiglitazone, 50 µM muraglitazar and 300 µM tesaglitazar represented around 1-, 8-, 50-, 35- and > 400-fold the therapeutic concentrations respectively (Miller and Spence, 1998; Ericsson *et al.*, 2004; Wang *et al.*, 2006; Scheen, 2007).

Despite its relatively low expression level in the liver, PPARγ is thought to be critical in the development of hepatic steatosis (Desvergne *et al.*, 2006). However, the role of PPARγ in NASH remains controversial. Indeed, this nuclear receptor is generally overexpressed in steatotic liver and has been reported to have a pro-steatogenic effect in both human and mouse hepatocytes (Moran-Salvador *et al.,* 2011). Rosiglitazone was found to induce accumulation of neutral lipids, as detected by Oil Red O staining in the cytoplasm of primary hepatocytes isolated from control mice, an effect that was markedly amplified by association of rosiglitazone and OA (Moya *et al., 2010*; Moran-Salvador *et al.,* 2011). By contrast, our results showed a reduction in steatosis in HepaRG cells after a 2-week treatment with rosiglitazone, which is in agreement with *in vivo* observations. Indeed, clinical studies have

reported a reduction of steatosis after several months of treatment with rosiglitazone (Ratziu *et al.*, 2008) and pioglitazone (Belfort *et al.*, 2006). However, a systematic review and meta-analysis of the effects of thiazolidinediones have emphasized only their modest effect in the improvement of patients with NASH but at the cost of significant weight gain and other adverse events (Musso *et al., 2010*; Mahady *et al.,* 2011). Taken altogether, such variable data could be explained by species differences, additional *in vivo* effects of glitazones in non-hepatic tissues and the use of different experimental conditions *in vitro*.

The dual PPARα/γ agonists muraglitazar and to a lesser degree tesaglitazar, also ameliorated steatosis in HepaRG cells. Muraglitazar had even a stronger effect than rosiglitazone on the decrease in triglycerides content and induction of FAO, that could be related to its higher affinity for PPARγ (Fievet *et al.*, 2006). Both glitazars have been reported to improve steatosis *in vivo*. A recent study on the effects of muraglitazar on glucose and lipid metabolism or fat distribution in patients has shown a beneficial effect on fat content, similar to that observed with pioglitazone (Fernandez *et al.,* 2011). Tesaglitazar has been reported to ameliorate NAFLD in diabetic, low density receptor-deficient mice (Zhang *et al.,* 2004) and to improve lipid profile in dyslipidemic patients (Tonstad *et al.*, 2007). These two dual PPAR agonists were attractive as therapy for NASH and the metabolic syndrome. Unfortunately, due to cardiac or renal toxicities, muraglitazar and tesaglitazar were stopped during clinical trials. However, our results bring further support towards on-going development of new glitazars, such as cevoglitazar (Chen *et al.,* 2010) and aleglitazar (Deehan *et al.,* 2012).

Fenofibrate, a PPARα agonist and bezafibrate, a PPAR pan-agonist, were also tested on steatotic HepaRG cells. In agreement with *in vivo* studies (Tonstad *et al.*, 2007; Musso *et al.*, 2010; Fernandez *et al.*, 2011; Mahady *et al.*, 2011; Lomonaco *et al.*, 2013), they were found to have less beneficial effects than rosiglitazone and glitazars on steatotic HepaRG cells. Indeed, despite their effectiveness in animal models (Tanaka *et al.*, 2003; George and Liddle, 2008) PPARα agonists have been disappointing in clinical trials. Bezafibrate has been reported to decrease hepatic steatosis and to increase expression of enzymes involved in FAO in mice (Nagasawa *et al.*, 2006). For this drug, an involvement of PPARβ/δ is likely since activation of PPARβ/δ has also been reported to deplete lipid accumulation in mice (Wang *et al.*, 2003). By contrast, no impact on steatosis was obtained in patients after a 12-month-treatment with clofibrate, another PPARα agonist (Laurin *et al.*, 1996).

To better understand the mechanisms involved in the decrease of triglycerides content by PPAR agonists, expression of several genes involved in the main lipid metabolism functions was analysed. Since HepaRG cells were simultaneously exposed to OA and PPAR agonists, both pro-steatogenic and anti-steatogenic effects could be expected at the level of gene expression. The PPARγ agonist rosiglitazone and especially the dual PPAR agonist muraglitazar, which were the most potent compounds in reducing triglycerides content and inducing FAO in HepaRG cells also exerted the highest effects on deregulation of several key genes involved in lipid metabolism, including ACOX1, CPT1A, PPARA, PPARG, ADFP, PLIN4, LXRA, FXR and CAR. ACOX1 and CPT1A were dramatically up-regulated from 1-day and remained expressed at high levels after 14-day treatment, especially with muraglitazar. ACOX1 enzyme, which is the first and rate-limiting enzyme of the peroxisomal β-oxidation pathway, plays a major role in the development of hepatic steatosis in rodents (Fan *et al.*, 1996) and re-expression of ACOX1 isoform in ACOX1$^{-/-}$ mice leads to reversal of hepatic steatosis (Vluggens *et al.,* 2010). Peroxisomal β-oxidation is critical for the degradation of very long chain fatty acids that are not able to be degraded by the mitochondrial β-oxidation system. After shortening of these long-chain fatty acids, they are taken-up by the mitochondrial system, in which CPT1A is a major enzyme involved.

Transcriptomic modulations were confirmed by investigation of FAO activity after treatments of OA-overloaded HepaRG cells with PPAR agonists. Indeed FAO activity was increased after 14-day repeat treatment with all PPAR agonists, especially with muraglitazar, supporting the view that the diminution of steatosis in HepaRG cells might be explained by a sustained activation of FAO.

It is noteworthy that expression of genes encoding perilipin proteins, such as ADFP and PLIN4, implicated in lipid droplet formation was also up-regulated by PPAR agonist treatment. These two proteins are well known to coat the lipid droplets, and consequently they represent a marker of steatosis. Consequently, a decreased expression of ADFP and PLIN4 would have been expected in cells treated with PPAR agonists due to the reversal of steatosis and diminution of triglycerides content. However, according to recent data, these two perilipin proteins are thought to protect non adipose cells, such as liver cells, from the lipotoxicity of free fatty acids by vesicle formation (Borg *et al.*, 2009; Simard *et al.,* 2010). This could explain the persistent over-expression of those perilipin proteins in steatotic HepaRG cells.

As emphasized above, muraglitazar was the most effective PPAR agonist to reverse steatosis in HepaRG hepatocytes overloaded with OA. Analysis of the deregulated genes including those encoding nuclear receptors, such as LXRα, FXR and CAR, suggested that muraglitazar could reverse steatosis by modulation of expression of these genes. The absence of significant diminution of LXRα transcripts supported the view that muraglitazar had no direct effect on LXRα expression. However, activation and overexpression of FXR by this compound might result in inhibition of LXRα action, that in turn would lead to the diminution of LXRα target genes, such as SREBP1-c, with as a consequence a reduction of *de novo* lipogenesis in accordance with diverse recent studies showing that FXR activation could reduce LXRα effect and then down-regulate *de novo* lipogenesis (Watanabe *et al.*, 2004; Zhang *et al.*, 2004).

In addition, muraglitazar still induced CAR expression after a 14-day treatment that could also result in down-regulation of SREBP1c and stimulate the FAO pathway (Dong *et al.*, 2009). This result could be an explanation why muraglitazar was the most potent PPAR agonist in reducing steatosis in HepaRG cells. Further investigations of the precise effects of the different classes of PPAR agonists on nuclear receptors and the resulting alterations of lipid metabolism should allow better characterizing the mechanisms by which these agonists differentially reverse steatosis.

In summary, this study represents the first analysis of the effects of diverse PPAR agonists on the different PPAR subtypes in steatotic human hepatocytes after repeat treatment. Its brings a proof of concept that drug-induced reversal of steatosis in man could be evaluated *in vitro* before conducting long-term and costly *in vivo* studies in animals and patients. In addition, our results show that although all PPAR agonists were effective dual PPARα/γ and PPARγ agonists appeared to be the most potent inducers of FAO and the most effective in reducing triglyceride accumulation in OA-overloaded HepaRG hepatocytes. These data give new insights in the effects of PPAR agonists on human hepatic steatosis and suggest that the HepaRG cell line appears suitable for a better understanding of the mechanisms involved in steatosis reversal, which is essential for the future development of novel and efficient therapeutic PPAR agonist agents for the treatment of NAFLD.

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FIGURE LEGENDS

Figure 1: Experimental design. Human HepaRG cells were cultured for two weeks (proliferative phase) with an appropriate medium as described in Material and Methods, and then the cells were transferred to the same medium supplemented with 2% DMSO in order to achieve cell differentiation. The cells were rendered steatotic after a 24 h treatment by oleic acid (OA); then they were treated for 1 day or 14 days with the PPAR agonists.

Figure 2: Determination of cell viability after PPAR agonist treatments in oleic acid-overloaded HepaRG cells. Cells were incubated for 24h with 250 µM oleic acid (OA) or the vehicle only (control) and then treated with PPAR agonists; *i.e.* 40 µM troglitazone (TRO), 50 µM rosiglitazone (ROSI), 50 µM muraglitazar (MURA), 300 µM tesaglitazar (TESA), 25 µM fenofibrate (FENO) or 25 µM bezafibrate (BEZA) for 1 day or every 2-3 days for 14 days. Cytotoxicity was measured by the MTT colorimetric assay. Each point is the mean ± SEM of three independent experiments. All results are expressed relative to untreated control, arbitrarily set at the value of 100%; ns: no significant

Figure 3: Determination of neutral lipid accumulation by Oil Red O staining after PPARs agonist treatments in oleic acid-overloaded HepaRG cells. (A) Cells were incubated for 24h with 250 µM oleic acid (OA) or the vehicle only (control) and then treated with PPAR agonists; *i.e.* 40 µM troglitazone (TRO), 50 µM rosiglitazone (ROSI), 50 µM muraglitazar (MURA), 300 µM tesaglitazar (TESA), 25 µM fenofibrate (FENO) or 25 µM bezafibrate (BEZA) for 1 day or every 2-3 days for 14 days. Neutral lipids (triglycerides and cholesterol esters) were stained by Oil Red O and the cells were photographed with phase-contrast microscope. Magnification 20X. **(B)** Quantification of Oil Red O pictures was realized by ImageJ software; areas of stained lipid droplets were determined for each condition (3 pictures per condition) and normalized by the areas of hepatocyte clusters. Each point is the mean ± SEM of three independent experiments. All results are expressed relative to untreated control, arbitrarily set at the value of 100%. #$p<0.05$ compared with control, *$p<0.05$ compared with OA treatment.

Figure 4: Triglycerides quantification after PPAR agonist treatments in oleic acid-overloaded HepaRG cells. Cells were incubated for 24h with 250 µM oleic acid (OA) or the vehicle only (control) and then treated with PPAR agonists; *i.e.* 40 µM troglitazone (TRO), 50 µM rosiglitazone (ROSI), 50 µM muraglitazar (MURA), 300 µM tesaglitazar (TESA), 25 µM fenofibrate (FENO) or 25 µM bezafibrate (BEZA) for 1 day or every 2-3 days for 14 days. Triglycerides (TG) levels were measured by spectrophotometry after chloroform/methanol extraction. Each point is the mean ± SEM of three independent experiments. All results are expressed relative to untreated control, arbitrarily set at the value of 100%. #$p<0.05$ compared with control, *$p<0.05$ compared with OA treatment.

Figure 5: Determination of fatty acid oxidation after PPAR agonist treatments in oleic acid-overloaded HepaRG cells. Cells were incubated for 24h with 250 µM oleic acid (OA) or the vehicle only (control) and then treated with PPAR agonists; *i.e.* 40 µM troglitazone (TRO), 50 µM rosiglitazone (ROSI), 50 µM muraglitazar (MURA), 300 µM tesaglitazar (TESA), 25 µM fenofibrate (FENO) or 25 µM bezafibrate (BEZA) for 1 day or every 2-3 days for 14 days. Fatty acid oxidation (FAO) was evaluated by measuring [^{14}C]-labeled acid-soluble β-oxidation products generated by cells after a 90 minutes incubation with [^{14}C]-palmitic acid. Each point is the mean ± SEM of three independent experiments. All results are expressed relative to untreated control, arbitrarily set at the value of 100%. #$p<0.05$ compared with control, *$p<0.05$ compared with OA treatment.

TABLES

Table 1: Primer sequences for RT-qPCR

Gene	Name	Forward Primer	Reverse Primer
18S	18S	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC
ACADL	long-chain specific acyl-CoA dehydrogenase	GTCCAAACGTTTCGCTTCAT	TTTGGCAAAACAGTTGCTCA
ACOX1	acetyl-coA oxidase 1	ATGCCCAAGTGAAGATCCAG	GAAGATGAGGGAGTTTGGCA
ADFP	adipose differentiation-related protein	CTCATGGGTAGAGTGGAAAAGGAGCATTGG	TTGGATGTTGGACAGGAGGGTGTGGCACGT
ALB	albumin	TGCTTGAATGTGCTGATGACAGG	AAGGCAAGTCAGCAGGCATCTCATC
APOC3	apolipoprotein C3	GAACTGAAGCCATCGGTCAC	GGTTACATGAAGCACGCCAC
CAR	constitutive androstane receptor	TGATCAGCTGCAAGAGGAGA	AGGCCTAGCAACTTCGCATA
CPT1A	carnitine O-palmitoyltransferase 1 A	GCCTCGTATGTGAGGCAAAA	TCATCAAGAAATGTCGCACG
CPT2	carnitine O-palmitoyltransferase 2	TTTGGGTCAGGATTGAAAGC	TGGTTGCTCTGGACAAACAG
CYP2B6	cytochrome P450 2B6	TTCCTACTGCTTCCGTCTATCAAA	GTGCAGAATCCCACAGCTCA
CYP4A11	cytochrome P450 4A11	TATGACATCCTGAAGCCCTAT	GGTGAAAGGCATTCCTCACA
ECH1	enoyl CoA hydratase 1	GGTGATCTCTGGTGCAGGAAA	CTTGGGGCACCTCTCGATGAC
ELOVL6	elongation of very long chain fatty acids protein 6	ATTCATTAGGTGCCGACCAC	TTCGAAAAGCAGTTCAACGA
FASN	fatty acid synthase	AACTCCTGCAAGTTCTCCGA	GCTCCAGCCTCGCTCTC
FXR	farnesoid X receptor	GGTAGCAGAGATGCCTGTAACAA	CACAGCTCATCCCCTTTGATC
LPL	lipoprotein lipase	AATGAGGTGGCAAGTGTCCT	CTCCAGAGTCTGACCGCCT
LXRα	liver X receptors α	GATCGAGGTGATGCTTCTGGA	CAAGGCAAACTCGGCATCAT
HADHA	hydroxyacyl-CoA dehydrogenase A	CCGTTCCTCTGGAGGTTTTA	TGGTAGAAGCATTCGTGCAG
PLIN4	perilipin-4	CAGATGCAGGAAGCATCAAA	GCGACTAAAAGGCACTCTGG
PPARA	peroxisome proliferator-activated receptors alpha	CATTACGGAGTCCACGCGT	ACCAGCTTGAGTCGAATCGTT
PPARG	peroxisome proliferator-activated receptors gamma	GATGACAGCGACTTGGCAA	CTTCAATGGGCTTCACATTCA
PXR	pregnane X receptor	CCAGGACATACACCCCTTTG	CTACCTGTGATGCCGAACAA

SCD	acyl-CoA desaturase	GACGATGAGCTCCTGCTGTT	CTCTGCTACACTTGGGAGCC
SREBP1	sterol regulatory element-binding protein 1	AGGGAAGTCACTGTCTTGGTTG	CTGCTGACCGACATCGAA
THRSP	thyroid hormone-inducible hepatic protein	AGGCCTTTCTGCTCTCATCA	AAATGACGGGACAAGTTTGG

Table 2: Expression of mRNAs encoding genes related to lipid metabolism, nuclear receptors and liver functions after PPAR agonist treatments in oleic acid-overloaded HepaRG cells. Cells were incubated for 24h with 250 µM oleic acid (OA) or untreated (control) and then treated with PPAR agonists; i.e. 40 µM troglitazone (TRO), 50 µM rosiglitazone (ROSI), 50 µM muraglitazar (MURA), 300 µM tesaglitazar (TESA), 25 µM fenofibrate (FENO) or 25 µM bezafibrate (BEZA) for 1 day or every 2-3 days for 14 days. mRNAs were analysed by RT-qPCR. Results are expressed as fold of the value found in control cells arbitrarily set at 1. Data are mean ± SEM of three independent experiments. Data with $p<0.05$ compared with control are indicated in bold and *$p<0.05$ compared with OA treatment.

		1 Day						
		OA	OA+TRO	OA+ROSI	OA+MURA	OA+TESA	OA+FENO	OA+BEZA
De novo Lipogenesis	ELOVL6	1.12±0.19	1.41±0.21	**1.88±0.03***	**2.04±0.36**	**2.73±0.69***	**2.66±0.53***	**1.98±0.18***
	FASN	0.88±0.06	**0.75±0.04**	0.96±0.17	1.04±0.27	0.87±0.12	1.57±0.28*	**1.77±0.18***
	PPARG	1.31±0.20	1.29±0.24	1.35±0.30	1.67±0.42	**1.74±0.20**	**2.67±0.49***	1.62±0.27
	SCD1	1.14±0.19	1.09±0.14	1.05±0.20	**2.14±0.20***	2.35±1.14	**3.60±0.74***	**2.43±0.30***
	SREBP1	1.48±0.13	**2.22±0.35**	**1.57±0.16**	1.83±0.37	1.39±0.30	**1.73±0.11**	**1.68±0.16**
	THRSP	1.33±0.18	0.97±0.12	1.29±0.20	2.10±0.88	1.42±0.35	**0.63±0.16***	**0.49±0.10***
Fatty acid oxidation and mitochondrial biogenesis	ACADL	**1.65±0.10**	**2.00±0.47**	**2.69±0.78**	1.48±0.35	1.42±0.42	**2.77±0.70**	1.86±0.38
	ACOX1	1.30±0.36	**1.89±0.15**	**2.55±0.68**	**5.08±1.11***	**2.43±0.47***	1.49±0.58	1.62±0.41
	CPT1A	**2.07±0.15**	**2.61±0.59**	**4.53±0.03***	**8.57±1.94***	**2.69±0.28***	**5.42±1.18***	**6.32±1.16***
	CPT2	1.14±0.15	1.42±0.19	**2.17±0.49***	**3.45±0.73***	**3.67±0.76***	**3.29±0.87***	**2.85±0.23***
	CYP4A11	1.02±0.08	**1.54±0.21**	1.62±0.41	**2.91±0.21***	1.33±0.24	1.12±0.53	1.27±0.17
	HADHA	1.20±0.17	1.36±0.17	**2.2±0.07***	**3.67±0.63***	1.91±0.67	**3.71±0.95***	**3.31±0.29***
	ECH1	1.01±0.12	**1.60±0.12***	**2.01±0.23***	**6.14±0.46***	**1.89±0.08***	1.21±0.58	1.17±0.37
	PPARA	1.43±0.36	1.08±0.10	1.29±0.25	**1.60±0.14**	1.27±0.19	**2.61±0.60***	1.35±0.35
Lipid hydrolysis and formation of droplets	ADFP	**2.97±0.71**	**2.23±0.58**	**6.28±0.07***	**7.59±1.09***	**8.47±1.74***	**4.84±1.61**	**5.93±0.46***
	LPL	1.15±0.12	**2.23±0.46**	**1.76±0.18**	1.56±0.29	1.62±0.53	1.72±0.28	1.41±0.20
	PLIN4	**2.07±0.44**	**3.88±1.18**	**5.48±2.02**	**8.90±3.17***	**4.43±1.14**	**5.83±2.73**	**7.43±1.28***
Lipoprotein	APOC3	0.99±0.07	1.08±0.09	1.03±0.24	1.31±0.21	1.65±0.34	1.92±0.31	1.34±0.14
Nuclear Receptors	LXRα	1.02±0.03	**2.23±0.29***	**2.32±0.20***	**1.50±0.11***	**1.95±0.13***	**1.74±0.13***	**1.54±0.25**
	FXR	1.01±0.10	1.05±0.04	1.43±0.10	**1.82±0.09***	1.39±0.11	1.20±0.09	**2.84±0.16***
	PXR	0.99±0.15	1.22±0.15	1.00±0.08	**0.76±0.08**	0.83±0.04	0.88±0.07	1.07±0.08
	CAR	1.00±0.01	**2.17±0.17***	1.26±0.04	**1.52±0.05***	0.96±0.06	**2.81±0.16***	**1.34±0.09**
Liver-specific proteins	ALB	1.21±0.17	0.95±0.12	1.12±0.27	1.41±0.04	1.43±0.47	2.01±0.43	1.54±0.25
	CYP2B6	1.30±0.20	**2.36±0.79**	**3.10±1.01***	1.16±0.53	1.45±0.59	1.40±0.39	0.76±0.14

		14 Days						
		OA	OA+TRO	OA+ROSI	OA+MURA	OA+TESA	OA+FENO	OA+BEZA
De novo Lipogenesis	ELOVL6	1.29±0.28	0.98±0.01	1.13±0.25	0.93±0.22	1.31±0.18	**0.63±0.09***	0.81±0.18
	FASN	0.99±0.08	0.88±0.05	1.05±0.08	0.91±0.24	0.93±0.01	**0.57±0.07***	0.62±0.18
	PPARG	1.03±0.16	0.98±0.08	1.07±0.13	0.87±0.12	1.03±0.12	**0.68±0.07**	0.74±0.21
	SCD1	1.11±0.19	1.16±0.20	1.26±0.14	1.38±0.46	1.18±0.29	0.77±0.10	0.94±0.18
	SREBP1	1.09±0.18	0.99±0.17	1.16±0.18	1.06±0.19	1.09±0.12	0.84±0.20	**0.68±0.13**
	THRSP	0.69±0.12	0.88±0.23	**0.72±0.02**	**0.69±0.08**	0.85±0.13	0.82±0.20	1.10±0.50
Fatty acid oxidation and mitochondrial biogenesis	ACADL	1.06±0.19	1.19±0.15	1.42±0.14	1.38±0.36	1.19±0.15	**0.65±0.03***	0.69±0.12
	ACOX1	1.36±0.06	1.58±0.30	**2.00±0.14***	**2.5±0.27***	**2.19±0.25***	**0.57±0.17***	1.08±0.63
	CPT1A	**1.74±0.09**	**2.16±0.37**	**3.46±0.59***	**5.02±1.63***	**2.72±0.20***	**2.23±0.59***	**3.06±0.46***
	CPT2	1.05±0.06	1.15±0.15	0.99±0.13	0.97±0.12	1.15±0.18	0.91±0.08	1.22±0.59
	CYP4A11	0.98±0.06	1.31±0.47	1.37±0.42	1.21±0.34	1.11±0.11	0.98±0.24	0.95±0.12
	HADHA	1.13±0.04	1.31±0.49	**1.63±0.21***	**1.96±0.56***	**1.74±0.03***	1.01±0.2	1.60±0.69
	ECH1	0.95±0.07	1.21±0.21	1.51±0.57	**2.05±0.45***	**1.61±0.09***	0.99±0.18	0.97±0.39
	PPARA	0.90±0.04	0.64±0.16	0.75±0.13	**0.53±0.01***	1.03±0.18	**0.68±0.09***	0.67±0.20
Lipid hydrolysis and formation of droplets	ADFP	**1.92±0.17**	**2.55±0.15***	**3.78±0.66***	**4.51±0.70***	**4.00±0.97***	1.49±0.33	**2.88±1.21**
	LPL	1.05±0.09	0.99±0.08	0.97±0.09	0.93±0.12	1.00±0.21	0.84±0.10	0.79±0.31
	PLIN4	**1.62±0.10**	**5.46±0.63***	**7.12±0.29***	**11.42±2.17***	**6.37±0.34***	**1.96±0.19**	**3.78±0.68***
Lipoprotein	APOC3	1.14±0.04	1.40±0.24	**1.54±0.09***	**1.72±0.18***	**1.61±0.01***	0.82±0.05	0.81±0.16
Nuclear Receptors	LXRα	1.01±0.05	**0.58±0.10***	1.30±0.08	1.21±0.09	**0.71±0.09***	**0.66±0.03***	1.39±0.09
	FXR	1.12±0.06	1.40±0.26	1.27±0.03	**2.10±0.08***	1.41±0.08	**0.35±0.09***	0.72±0.04
	PXR	1.01±0.08	**0.54±0.01***	0.94±0.08	**1.52±0.08***	**0.71±0.04***	**0.75±0.03**	**1.47±0.04***
	CAR	1.00±0.06	**0.65±0.03***	**0.66±0.04***	**2.26±0.08***	**0.81±0.01**	**0.81±0.03**	1.47±0.23
Liver-specific proteins	ALB	1.02±0.14	**1.82±0.34**	**1.61±0.12***	**1.90±0.27***	**1.64±0.06***	**0.69±0.06**	0.72±0.18
	CYP2B6	1.30±0.34	**3.30±0.47***	**1.77±0.23**	2.43±1.89	1.03±0.26	1.14±0.15	1.03±0.21

Figure 1
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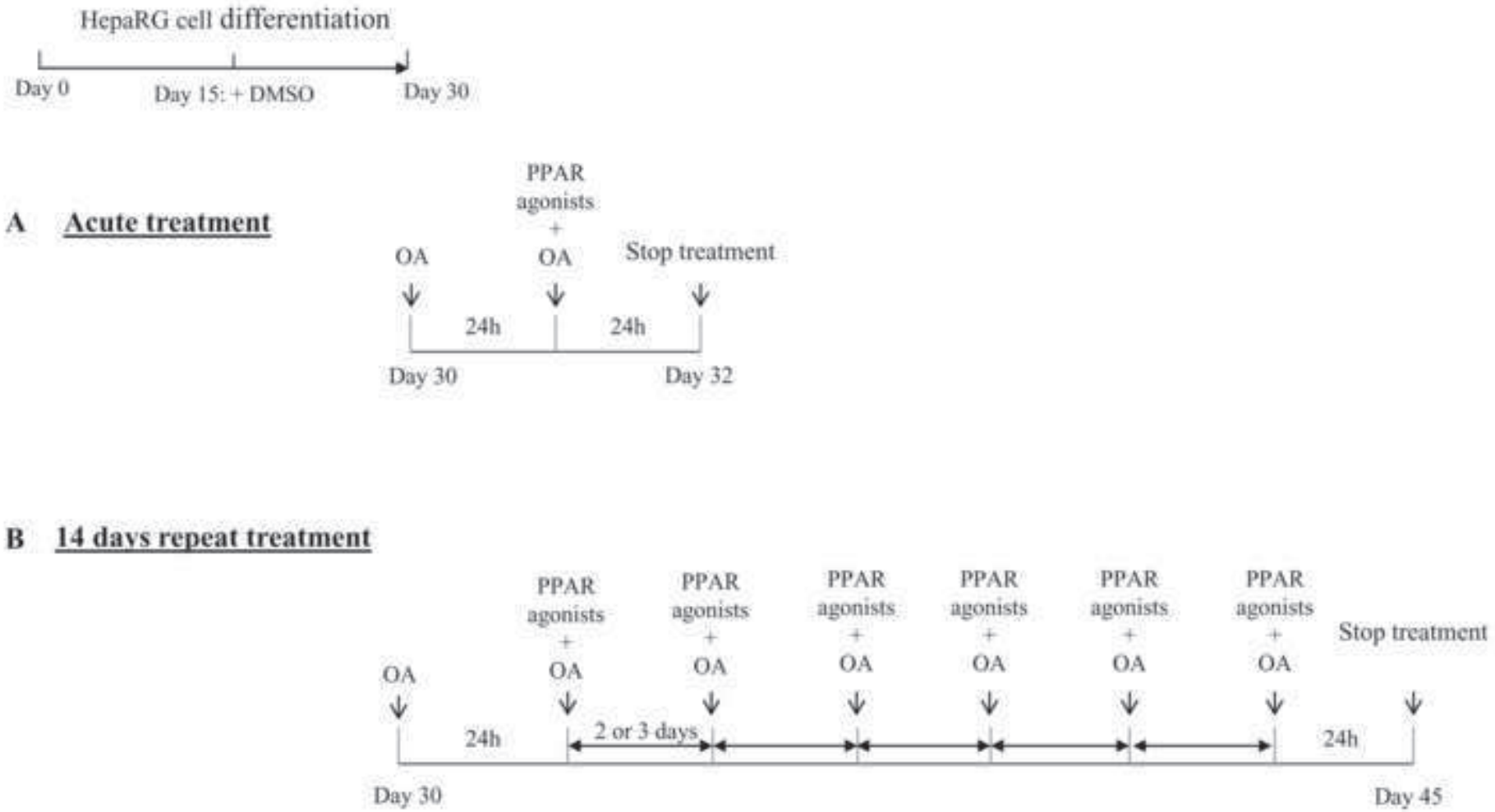

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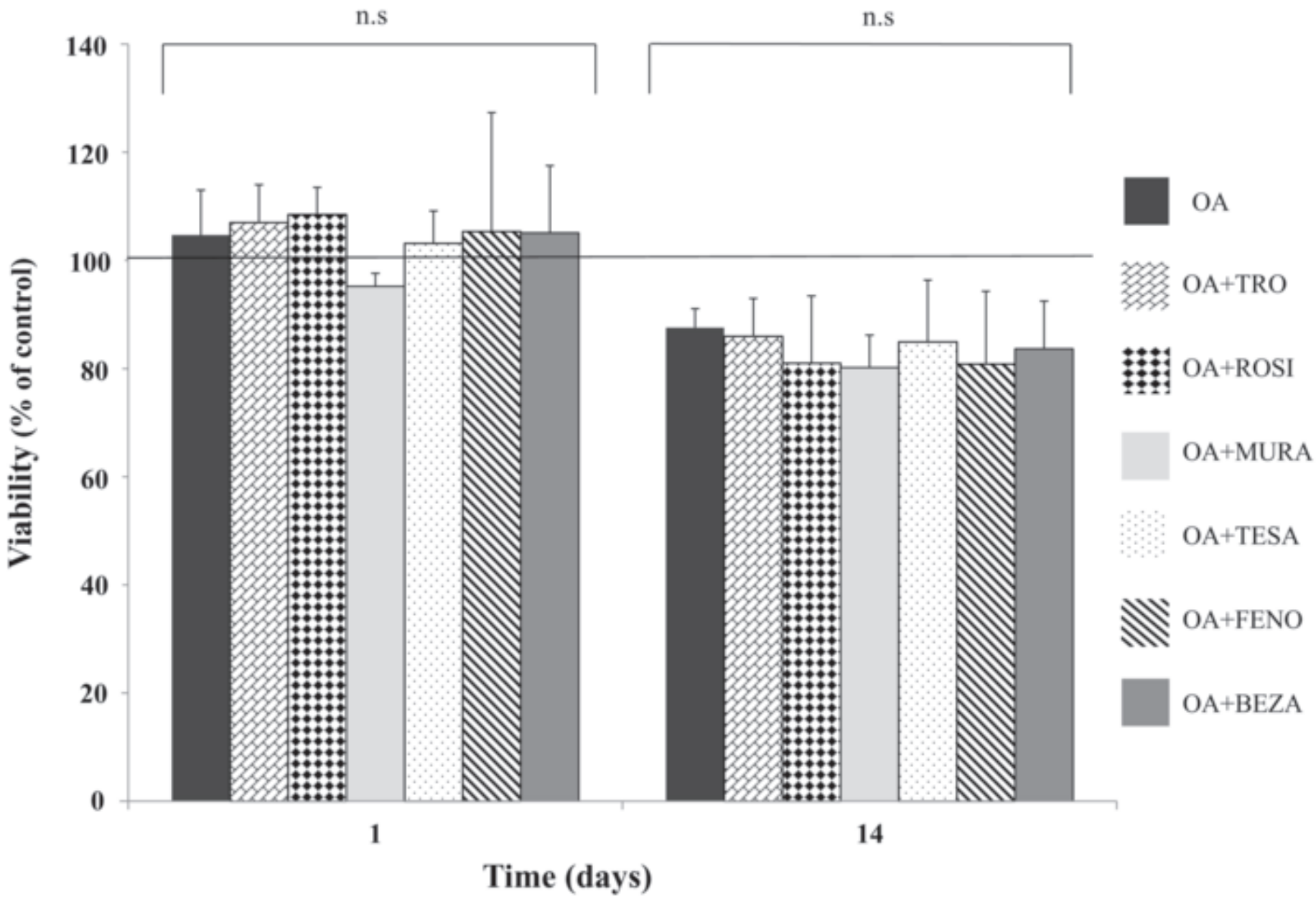

Figure 3-A
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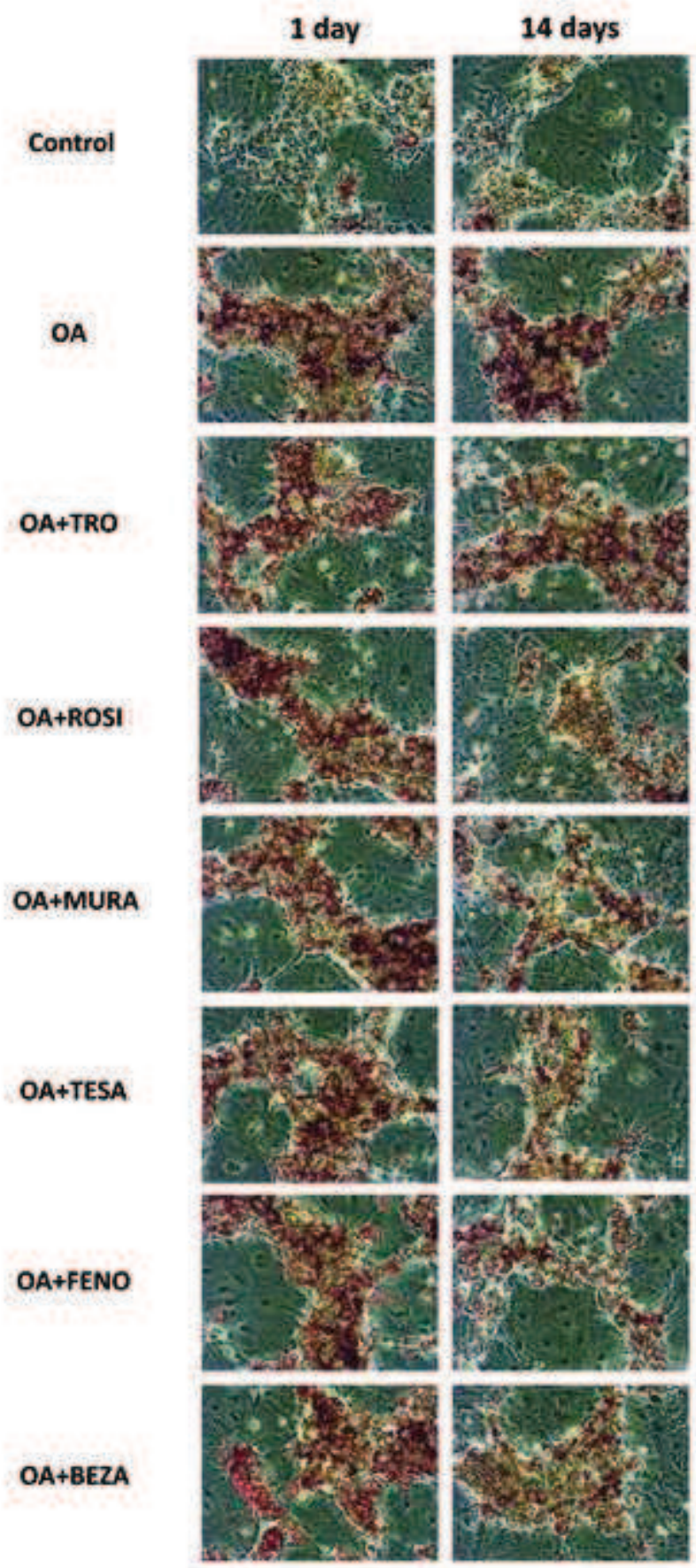

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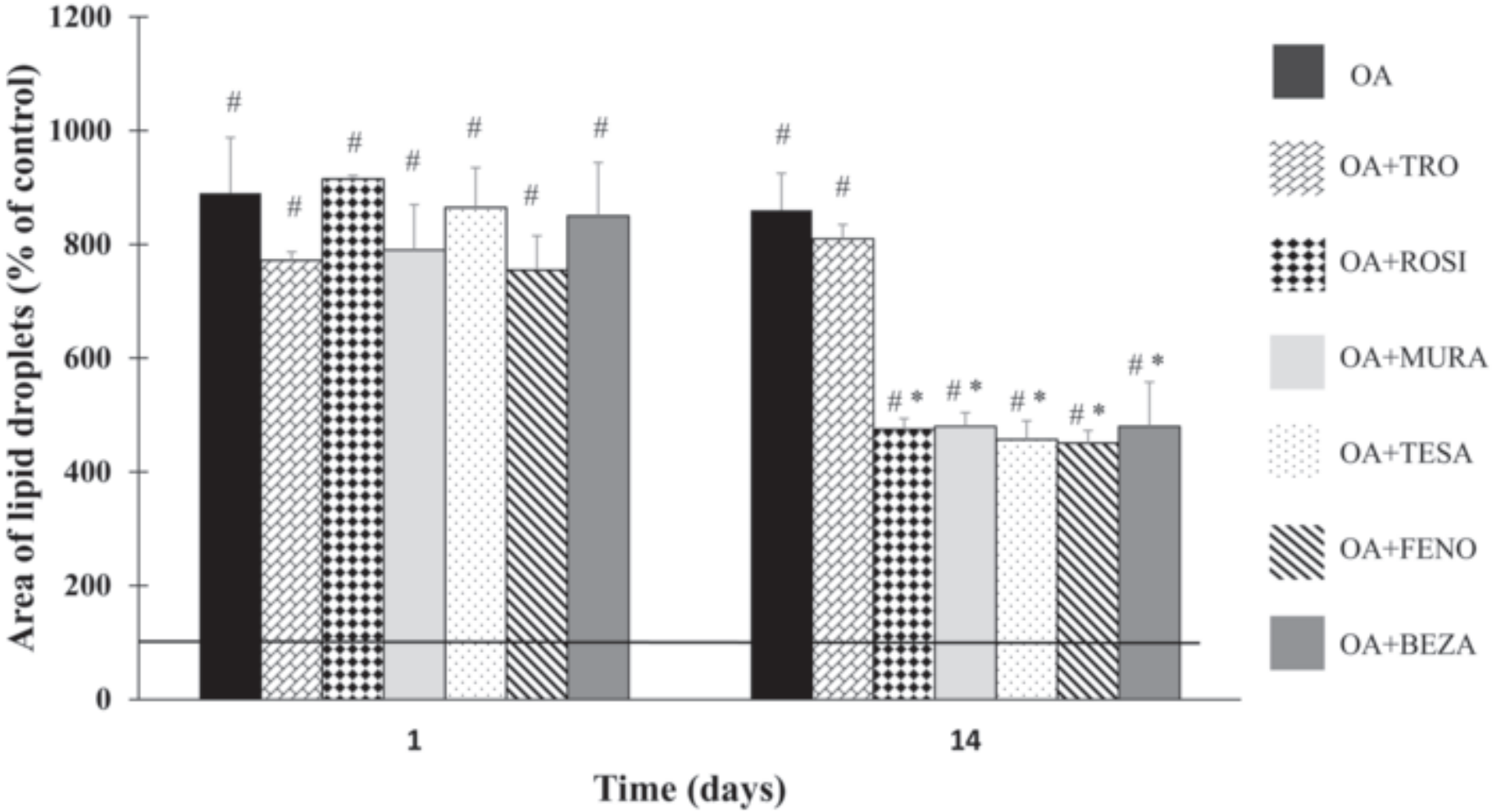

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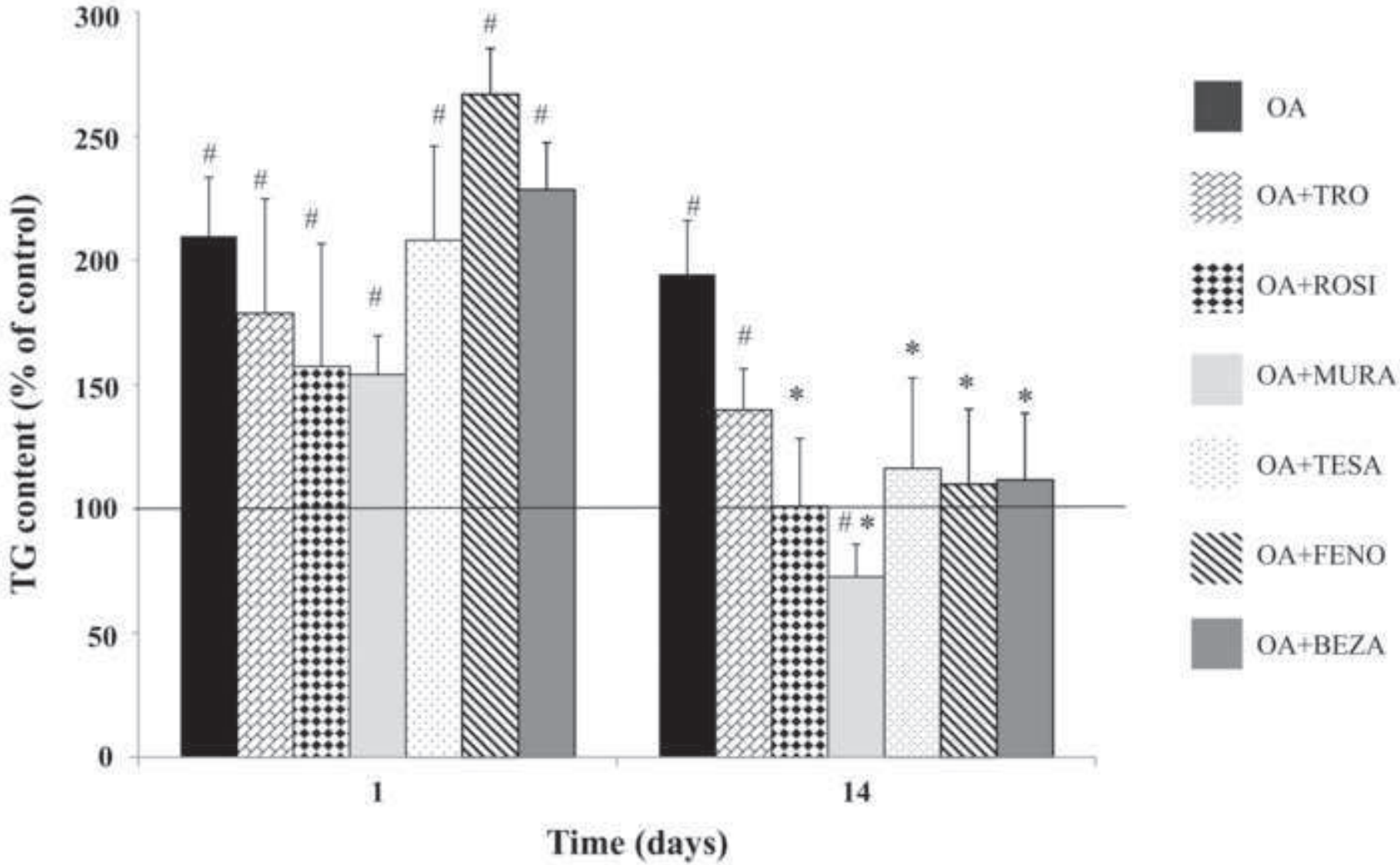

Figure 5

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