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Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3T3-L1 cells

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Abstract

Aims:

Oleuropein and hydroxytyrosol, which are antioxidant molecules found in olive leaves and oil, have been reported to exert several biochemical and pharmacological effects. These polyphenols are able to prevent low-density lipoprotein oxidation and protect cells against several diseases. Here, we studied the effect of these compounds on adipocyte differentiation in 3T3-L1.

Main Methods:

To perform this study, 3T3-L1 preadipocytes viability was analysed via Trypan blue and MTT assays, and triglycerides were stained with Oil Red O. Adipogenesis releated genes expression were checked by RT-PCR and qRT-PCR. Also, cells counting and flow cytometry were used to analyse the mitotic cell cycle during the adipogenesis clonal expansion phase.

Results:

Oleuropein and hydroxytyrosol dose-dependently suppressed intracellular triglyceride accumulation during adipocyte differentiation without effect on cell viability. PPARγ, C/EBPα and SREBP-1c transcription factors and their downstream targets genes (GLUT4, CD36 and FASN) were down-regulated after treatment by oleuropein and hydroxytyrosol. At 200 and 300 μmol/L oleuropein or 100 and 150 μmol/L hydroxytyrosol, the greatest effect on the adipogenesis process was observed during the early stages of differentiation. Flow cytometry revealed both polyphenols to inhibit the division of 3T3-L1 preadipocytes during mitotic clonal expansion and cause cell cycle delay. Furthermore, oleuropein and its derivate hydroxytyrosol decreased the transcriptional activity of SREBP-1c in a stable transfected 3T3-L1 cell line.

Significance:

These findings indicate that both compounds are able to prevent 3T3-L1 differentiation by inhibition of the mitotic clonal expansion and downregulation of the adipogenesis related genes.

Keywords: adipogenesis, polyphenols, olive leaves, SREBP-1c, cell cycle.

Introduction

Obesity, a complex disorder with multiple causes that include both genetic and environmental factors, is a major health problem in both developed and developing countries. At the cell biological level, obesity is characterised by an increase in the number and size of adipocytes in adipose tissue, and leads to the development of type II diabetes mellitus, cardiovascular disease and hyperlipidemia (Spiegelman and Flier 1996; Saltiel and Kahn 2001). Several antiobesity mechanisms have thus far been proposed: reduction of energy and food intake, decreased preadipocyte proliferation and differentiation, and increased lipolysis and fat oxidation. Current studies on obesity focus on discovering food ingredients that have the capability to suppress the proliferation and the differentiation of adipocytes in adipose tissues (Lee et al. 2008; Kim et al. 2010; Yang et al. 2006; Maeda et al. 2006).

Phenolic compounds, which are secondary plant products, are consumed regularly as part of the human diet and are associated with the prevention of some diseases. Mediterranean diets are associated with lower mortality from cardiovascular disease and cancers (Trichopoulou et al. 2003). Olive tree products are known to be the main source of healthy Mediterranean diet ingredients due to their high phenolic content (Visioli et al. 2002). The phenolic compounds in olive oil and leaves are a complex mixture of secoiridoid derivates that include hydroxytyrosol, tyrosol, hydroxytyrosol acetate and other benzoic and cinnamic acid derivatives (Mateos et al. 2001; Litridou et al. 1997). Oleuropein appears to be the principal phenolic compound in olive oil and leaves (Fig. 1). Its concentration varies with cultivar and climate and is several times higher in the olive leaf than the oil (Ryan et al. 2002). On hydrolysis, oleuropein can produce other bioactive substances, including elenolic acid and hydroxytyrosol (Fig. 1). Several in vitro and in vivo studies have shown that oleuropein and its derivate hydroxytyrosol possess a wide range of biochemical and pharmacological properties. In fact, oleuropein is able to inhibit hyperglycemia and oxidative stress induced in diabetic rabbits, increase the resistance of LDLs to oxidation, inhibit cell proliferation and induce cell apoptosis in MCF-7 cancer cells, and enhance osteoblastogenesis and inhibit adipogenesis in stem cells derived from human bone marrow (Al-Azzawie and Alhamdani 2006; Han et al. 2009; Santiago-Mora et al. 2010). In addition, hydroxytyrosol, regarded as the most potent antioxidant in olive leaves and oil phenolic fraction, provides *in vitro* protection of human hepatoma cells (HepG2) against oxidative stress, inhibits the cell cycle progression in HL60 and MCF-7 cells and reduces *in vivo* serum levels of total cholesterol, triglycerides and LDL when administered to rats fed a cholesterol-rich diet (Han et al. 2009; Fabiani et al. 2008; Goya et al. 2007; Gonzalez-Santiago et al. 2006).

To explore the possibility that oleuropein and hydroxytyrosol might inhibit *in vitro* adipocyte differentiation, we carried out the following experiments to determine the effects of both phenolic compounds on the adipogenesis and differentiation of 3T3-L1 cells.

Materials and methods

Materials. 3T3-L1 cells were provided from the Health Science Research Resources Bank (HSRRB, Osaka, Japan). Oleuropein was purchased from Extrasynthese Company (Genay, France). Hydroxytyrosol was obtained from Cayman Chemical Company (Michigan, USA). Dulbecco's modified Eagle's medium (DMEM high-glucose), Dexamethasone, 3-iso-butyl-1-methylxanthine, and Insulin were purchased from Sigma-Aldrich (Missouri, USA).

Cell culture. 3T3-L1 cells were cultured in DMEM medium containing 10% FBS at 37 °C and 5% CO_2. Cells were plated at a density of 3×10^5 cells in 60 mm culture dish, and 5×10^4 in 24 wells plate. After reaching the confluence, adipocyte's differentiation was initiated using the same medium containing 10 mg/L insulin, 0.5 *m*mol/L isobutylmethylxanthine, and 1 μmol/L dexamethasone for 2 days. The medium was then replaced with DMEM containing 5 mg/L insulin for more 2 days, and then changed to fresh medium every 2 days. Hydroxytyrosol was diluted in ethanol, the final quantity of the solvent was 0.1% for control and treated cells for all experiment. Oleuropein was dissolved in dDW water and directly diluted in DMEM medium.

Oil red O staining and quantification. After differentiation, 3T3-L1 cells were washed twice with phosphate buffered saline (PBS, pH 7.4), fixed with 4% paraformaldehyde (Kantou Chemistry, Tokyo, Japan) at 4 °C for 1 h, and then stained with 3 g/L Oil red O (in 60% isopropanol) at room temperature for 10 min. Cells were washed exhaustively with sterile water, and pictures were taken using a microscope (BioZero BZ-8000; Keyence, Osaka, Japan). Moreover, differentiated adipocytes were stained with 0.3 g/L Oil Red O, the dye was extracted with isopropanol, and the absorbance (OD. 420 nm) was measured by a Spectra Max microplate reader (Spectra Max 190; Molecular Devices Corporation, CA, USA).

Triglyceride assay. 3T3-L1 cells were rinsed twice with PBS buffer and lysed in 50 *m*mol/L Tris-HCL [pH: 6.8], 2% SDS and 6% β-mercapthoethanol. Total fat was extracted according to Bligh and Dyer method (Bligh and Dyer 1959). The cell extract (400 µL) was incubated

with 1 mL methanol and 0.5 mL chloroform for 2 h, and then 0.5 mL chloroform and 0.5 mL of sterile water were added, centrifuged briefly to collect chloroform phase. This extract was dried for overnight, and was dissolved in 10% triton-isopropanol solution. According to a manual of triglyceride E-test Wako (Wako, Osaka, Japan), the quantity of Triglyceride was measured. The quantity of triglycerides (μmol) was normalized by total protein content.

Measurement of GPDH activity. 3T3-L1 cells were differentiated on 60 mm culture dish for 8 days in the presence of hydroxytyrosol (0-150 μmol/L) or oleuropein (0-300 μmol/L). The cells were rinsed twice with PBS, and then scraped into 200 µL enzyme extract buffer (Sucrose 280 *m*mol/L, Tris-HCl 5 *m*mol/L [pH 8.0], EDTA 1 *m*mol/L, and β-mercapthoethanol 0.2%). Cells were sonicated and centrifuged at 15000×g at 4 °C for 10 min. The supernatant was collected to measure glycerol-3-phosphate dehydrogenase activity. The total protein quantity was quantified with protein assay kit (Bio-Rad laboratories, Inc., Tokyo, Japan).

MTT assay. 3T3-L1 cells were harvested in 24-well plate. After reaching the confluence, the culture medium was replaced by 500 µL containing hydroxytyrosol (0-200 μmol/L) or oleuropein (0-400 μmol/L), and the cells were incubated for further 48 hours. The culture medium was removed and replaced by 500 µL of fresh culture medium containing 10 % of sterile filtered MTT (Sigma-Aldrich). After 3 hours, the insoluble formazan crystals were dissolved in 500 µL/well isopropanol and absorbance was measured at 570nm against 630nm. The inhibition (%) was expressed as the percentage of viable cell compared to control.

Trypan Blue assay. 3T3-L1 cells were harvested for 2 days after confluence in the presence of hydroxytyrosol or oleuropein. Then, cells viability was quantified by Trypan Blue assay. After washing twice with PBS, cells were trypsinized and immediately stained with 0.5% trypan blue dye (Trypan Blue, Sigma-Aldrich) for 3 min. Cells were observed under an optical microscope, and the viability was calculated as the percentage ratio of the number of unstained cells relative to the total cells counted.

Fatty acid uptake assay. 3T3-L1 cells were differentiated with hydroxytyrosol or oleuropein in 60 mm plates. After preincubation for 2 h in serum-free DMEM, Fatty acid uptake was performed in 1 mL PBS (+) (NaCl 137 *m*mol/L, Na_2HPO_4 8.1 *m*mol/L, KCl 2.6 *m*mol/L, KH_2PO_4 1.47 *m*mol/L, $CaCl_2$ 0.9 *m*mol/L and $MgCl_2$ 0.33 *m*mol/L) containing 40 μmol/L

BODIPY 3823 and 20 μmol/L Albumin for 2 min in 37 °C. The uptake was stopped by washing twice with cold PBS (+), and fluorescent photos were taken using a fluorescence microscope (DMRXA, Leica Microsystems Inc, IL, USA) with L5 filter (505nm, Leica).

Glucose uptake assay. Differentiated 3T3-L1 cells were preincubated for 2 h in serum-free DMEM, and then incubated in KRH buffer (NaCl 131 *m*mol/L, KCl 4.7 *m*mol/L, $CaCl_2$ 2.5 *m*mol/L, NaH_2PO_4 2.5 *m*mol/L, MgSO4 1 *m*mol/L and HEPES 10 *m*mol/L) for 30 min. Uptake was then initiated by addition of 2-NBDG (Invitrogen Life Technologies, Carlsbad, CA, USA) at 50 μmol/L in KRP-H buffer. After 15 min, the reaction was stopped by a quick washing with cold KRP-H, and Fluorescence in the cells was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm with a Wallac ARVO SX 1420 multi-label counter (Perkin Elmer Life Sciences, Japan, Co. Ltd., Kanagawa, Japan).

Gene expression analysis. Total RNA was extracted from 3T3-L1 cells by acid-GTC-phenol method (Chomczynski and Sacchi 1987). After DNase I (Takara Bio, Otsu, Shiga, Japan) treatment and RNA repurification, the cDNA was synthesized using M-MLV Reserse Transcriptase (Takara), and subjected for PCR using the primers indicated in Table 1. Quantitative PCR analysis was carried out using SYBR Premix Ex Taq (Takara). Each cDNA was amplified (95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s, for 40 cycles) using specific primers (Table 2).

Flow cytometry analysis. Postconfluent 3T3-L1 cells were treated with DEX, IBMX and Insulin in the presence of several doses of oleuropein or hydroxytyrosol. Cells were tripsinized and fixed with 70% ethanol at 4°C for overnight. After removing of ethanol, cells were stained with propidium iodide (Sigma-Aldrich) for 30 min in the obscurity. Fluorscencent cells analysis was carried out by using Guava EasyCyte (Guava Technologies, Hayward, CA, USA).

Luciferase reporter assay of SREBP-1c transcription factor. To study the effect of hydroxytyrosol and oleuropein on the activity of SREBP-1c transcription factor, stable 3T3-L1 cells, transfected by a luciferase reporter plasmids that carry a SREBP-1c DNA-binding site (SRE-Luc), were constructed (Kim et al. 2010). The cells were plated at a density of 3×10^5 cells in 60 mm culture dish. After reaching the confluence, hydroxytyrosol (150 μmol/L) or oleuropein (300 μmol/L) were added for more 2 days. Cells were incubated with 400 µL

Repoter lisys buffer (Promega, Madison, WI, USA) for 60 min at -80 °C and then scraped. The lysate was centrifuged for 5 min at 15000×g, supernatant was collected and luciferase activity was measured with a Luminometer Micro Lumat LB96p (Berthold Technology, Bad Wildbad, Germany).

Results

Effect of hydroxytyrosol and oleuropein on 3T3-L1 differentiation

3T3-L1 cells were differentiated for 8 days in the presence of hydroxytyrosol or oleuropein. Totally differentiated adipocytes were stained and total lipid accumulation was quantified by Oil red O. As shown in Figure 2A, hydroxytyrosol and oleuropein inhibited most adipocyte differentiation at 150 μmol/L and 300 μmol/L respectively. Results from Oil red O quantification (Fig. 2B) showed that both hydroxytyrosol and oleuropein reduced total lipid content in a dose-dependent manner.

Effect of hydroxytyrosol and oleuropein on GPDH activity and triglyceride accumulation

GPDH enzyme occupies the central position in triglyceride synthesis and GPDH activity is evaluated as a differentiation marker for adipocytes. The effect of hydroxytyrosol and oleuropein on GPDH activity was examined. As displayed in Supplementary Figure 1A, hydroxytyrosol and oleuropein reduced GPDH activity in a dose-dependent manner. Cytosolic TG concentration was also dose-dependently decreased by hydroxytyrosol and oleuropein (Supplementary Fig. 1B.).

Effect of hydroxytyrosol and oleuropein on the viability of 3T3-L1 cells

3T3-L1 cells were incubated in the presence of hydroxytyrosol or oleuropein at various doses for 48 h. The percentage of viability was determined by Trypan Blue assay and MTT assay. As shown in Figures 3A and 3B, neither of these phenolic compounds had any effect on cell viability at the concentrations used during this study.

Time course effect of hydroxytyrosol and oleuropein on 3T3-L1 differentiation

To understand at which stage of differentiation hydroxytyrosol and oleuropein exhibited the most effect, 3T3-L1 cells were differentiated in the presence of several doses of hydroxytyrosol or oleuropein over 0-2 days (early stage), 2-4 days (middle stage), 4-6 days (late stage), and 0-8 days.

As shown in Figure 4, both hydroxytyrosol and oleuropein exhibited anti-adipogenic effects only in the early stage: their effect during the medium and late stages was very low, with no significant difference seen between the controls and the treated cells.

Effect of hydroxytyrosol and oleuropein on the gene expression of transcription factors PPARγ, C/EBPα, and their lipogenic target genes

To examine the mechanisms underlying hydroxytyrosol and oleuropein-induced suppression of 3T3-L1 differentiation, the expression of transcription factor PPARγ and C/EBPα, and their target genes were examined by RT-PCR and qRT-PCR. As shown in Figure 5A and Supplementary Fig 2, PPARγ and C/EBPα mRNA levels significantly decreased in a dose-dependent manner during adipocyte differentiation in cells treated with hydroxytyrosol or oleuropein. Since PPARγ and C/EBPα mRNA levels were decreased by hydroxytyrosol and oleuropein, we hypothesized that expression of their target genes may also be down-regulated. Indeed, under hydroxytyrosol and oleuropein treatment, the mRNA levels of CD36 and Glut4 were reduced in a dose-dependent manner during adipocyte differentiation (Fig. 5B and Supplementary Fig. 2).

Effect of hydroxytyrosol and oleuropein on glucose and fatty acid uptake

Because hydroxytyrosol and oleuropein downregulated the expression of GLUT4 and CD36 in a dose-dependent manner, we evaluated the glucose and fatty acid uptake in 3T3-L1 cells treated with both compounds. The uptake of 2-NBDG, a fluorescence-labeled glucose analog, was reduced in a dose-dependent manner after treatment with both phenolic compounds (Supplementary Fig. 3A).

Next, we evaluated the fatty acid uptake in 3T3-L1 adipocytes via using BODIPY3823. As shown in supplementary Figure 3B, hydroxytyrosol reduced the level of uptake of fatty acids at 150 μmol/L. The same effect was observed with oleuropein at 300 μmol/L.

Effect of hydroxytyrosol and oleuropein on mRNA expression and transcriptional activity of SREBP-1c

We used qRT-PCR to analyse the effect of hydroxytyrosol and oleuropein on the expression of the mRNA of SREBP-1c transcription factor and its target gene FASN. Expression of mRNA of the genes SREBP-1c and FASN decreased with increasing hydroxytyrosol or oleuropein doses in 3T3-L1 adipocytes (Fig. 6A and Supplementary Fig. 2).

To analyse the effect of hydroxytyrosol or oleuropein on SREBP1c transcriptional activity, 3T3-L1 cells were transfected with luciferase reporter plasmids that carry a SREBP-1c DNA-binding site (SRE-Luc) and exposed to hydroxytyrosol (150 μmol/L) or oleuropein (300 μmol/L). The transcriptional activity of SREBP1c decreased after treatment with both hydroxytyrosol and oleuropein (Fig. 6B), suggesting that hydroxytyrosol and oleuropein reduce fatty acid synthesis via inhibition of SREBP-1c in 3T3-L1 cells.

Effect of hydroxytyrosol and oleuropein on the clonal expansion and cell cycle progression of 3T3-L1 cells during the early stage of differentiation

As described above, hydroxytyrosol and oleuropein displayed their main effect during the early stage of differentiation. We thus anticipated that these polyphenols would affect the preadipocyte proliferation step. A cell number analysis after 24 h and 48 h revealed that hydroxytyrosol and oleuropein treatment inhibited DMI-induced clonal expansion and the cell number remained lower in the treated culture (Fig. 7)

We then examined the effect of both phenolic compounds on the cell cycle events during the early stage of differentiation. After DMI induction, preadipocyte cells simultaneously enter the cell cycle, resulting in the detection of dividing cells (G2/M) by flow cytometry analysis after 24 h of induction. On the other hand, hydroxytyrosol and oleuropein caused a significant delay in the progression of the cell cycle and increased G0/G1 and S population in a dose-dependent manner (Fig. 8)

Discussion

In our present study, we demonstrated that oleuropein and hydroxytyrosol inhibited the differentiation and adipogenesis of 3T3-L1 cells without affecting cell viability. Moreover, oleuropein and hydroxytyrosol reduced triglyceride accumulation, inhibited GPDH enzyme activity, downregulated the gene expression of the adipogenesis-related transcription factors PPARγ, C/EBPα and SREBP-1c, and affected the transcriptional activity of SREBP-1c.

Oleuropein and hydroxytyrosol suppressed lipid accumulation and GPDH enzyme activity in a dose-dependent manner. In fact, oleuropein inhibited triglyceride accumulation by around 40 and 70% at 200 and 300 μM respectively; with the same tendency, hydroxytyrosol inhibited triglyceride accumulation by around 55 and 70% at 100 and 150 μM respectively. The difference of efficiency of hydroxytyrosol and oleuropein against adipogenesis can be related to the difference of bioavailability of each one. As previously described,

Hydroxytyrosol had a polar structure giving it a high capability to across the membrane and exert its antioxidant effect in isolated rat aorta (Rietjens et al. 2007). Also, it was postulated that hydroxytyrosol adsorption is occurred via passive diffusion in Caco-2 cells (Manna et al. 2000). However, the hydrophilic sugar moiety in oleuropein probably prevents it from crossing the membrane and explained the poor bioavailability of this molecule. It has been proposed that oleuropein may diffuse through the lipid bilayer of the cell membrane and be absorbed via a glucose transporter (Manna et al. 2000; Edgecombe et al. 2000; Rietjens et al. 2006). Hao et al. (2010) reported that hydroxytyrosol over the concentration range of 0.1–10 μmol/L promoted mitochondrial biogenesis via stimulation of the transcriptional activity of PPARGC1α and its downstream targets genes in 3T3-L1 adipocytes but had no effect on triglyceride content or glycerol release during the adipogenesis process in the same cell lineage. The differentiation of 3T3-L1 adipocytes is regulated essentially by the action of PPAR and C/EBP families, and their downregulation reduced the maturation of 3T3-L1 preadipocytes (Tontonoz et al. 1994; Koutnikova et al. 2003; Rosen et al. 2002). Expression of PPARγ and C/EBPα markedly increased the expression of their downstream target genes involved in triacylglycerol metabolism including the fatty acid transporter CD36 and glucose transporter GLUT4. Our present study showed that oleuropein and hydroxytyrosol decreased the expression of PPARγ and C/EBPα and their downstream target genes CD36 and GLUT4 in a dose-dependent manner during the differentiation process. In addition, both phenolic compounds reduced glucose and fatty acid uptake in adipocytes after 8 days of treatment. In a recent study, oleuropein was found to reduce the expression of PPARγ and to inhibit adipogenesis in mesenchymal stem cells derived from human bone marrow (Santiago-Mora et al. 2010).

Time-course analysis of the effect of hydroxytyrosol and oleuropein showed these compounds to exhibit their strongest effects during the early stages of differentiation, which runs parallel to clonal expansion. Interestingly, hydroxytyrosol and oleuropein inhibited DMI-induced clonal expansion of 3T3-L1 cells and delayed the cell cycle progression in a dose-dependent manner. Our results are in agreement with several previous studies which showed that arresting or delaying the cell cycle of 3T3-L1 cells during the first 2 days of differentiation decreased cell number and inhibited the differentiation rate of adipocytes. Lee at al. (2009) concluded that reactive oxygen species facilitate adipocyte differentiation by accelerating cell cycle progression from the S to the G2/M phase, whereas the antioxidants genistein and resveratrol inhibit the differentiation of 3T3-L1 and delay the cell cycle progression during mitotic clonal expansion. Also, Vitisin A, a resveratrol tetramer, had the capability to inhibit

the differentiation of 3T3-L1 cells and blocked the cell cycle at the G1 to S phase transition (Kim et al. 2008).

Helix-loop-helix transcription factor SREBP-1c, a transcription factor that controls fatty acid synthesis, is an additional regulator of adipogenesis in parallel with C/EBPα and PPARγ pathways. SREBP-1c expression is significantly enhanced in 3T3-L1 adipocytes in response to insulin (Kim et al. 1998), and its transcriptional activity is increased under the stimulation of oxidative stress (Sekiya et al. 2008). The SREBP family has been found to directly regulate a group of genes involved in TG and cholesterol synthesis (Horton et al. 2003). In previous studies, dominant negative SREBP-1c expression was found to inhibit preadipocyte differentiation, and HLH overexpression to enhance the adipogenic activity of PPARγ (Kim and Spiegelman 1996). Many compounds have been described as inhibiting adipogenesis via SREBP-1c regulation (Izumi et al. 2009; Kim et al. 2010). Here, we demonstrated that oleuropein and hydroxytyrosol downregulated the expression of SREBP-1c and its downstream target gene (FASN) in a dose-dependent manner. Also, incubation of 3T3-L1 preadipocytes with oleuropein and hydroxytyrosol inhibited the transcriptional activity of SREBP-1c.

Conclusion

In conclusion, we suggest that oleuropein and hydroxytyrosol act on 3T3-L1 cells to reduce preadipocyte differentiation and lipid accumulation and thus regulate the size of fat cells, giving them potential as useful obesity-preventive additives to foods and drinks.

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References

Al-Azzawie HF, Alhamdani MS S. Hypoglycemic and antioxidant effect of oleuropein in alloxan-diabetic rabbits. Life Sci 2006;78:1371-7.

Bligh EG, Dyer WJ. A rapid method for total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-7.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chlorophorm extraction. Anal Biochem 1987;162:156-9.

Edgecombe SC, Strech GL, Hayball PJ. Oleuropein, an antioxidant polyphenol from olive oil, is poorly absorbed from isolated perfused rat intestine. J Nutr 2000; 130:2996-3002.

Fabiani R, Rosignoli P, De Bartolomeo A, Fuccelli R, Morozzi G. Inhibition of cell cycle pregression by hydroxytyrosol is associated with upregulation of cyclin-dependant protein kinase inhibitors $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ and with induction of differentiation in HL60 cells. J Nutr 2008;138:42-8.

Gonzalez-Santiago M, Martin-Bautista E, Carrero JJ, Fonolla J, Baro L, Bartolome MV, et al. One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development. Atherosclerosis 2006;188:35-42.

Goya L, Mateos R, Bravo L. Effect of the olive oil phenol hydroxytyrosol on human hepatoma HepG2 cells. Protection against oxidative stress induced by tert-butylhydroperoxide. Eur J Nutr 2007;46:70-8.

Han J, Talorete TPN, Yamada P, Isoda H. Anti-proliferative and apoptotic effects of oleuropein and hydroxytyrosol on human breast cancer MCF-7 cells. Cytotechnology 2009;59:45-53.

Hao J, Shena W, Yuc G, Jiaa H, Lia X, Fenga Z, et al. Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3-L1 adipocytes. J Nutr Biochem 2010;21:634-44

Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. Proc Natl Acad Sci USA 2003;100:12027-32.

Izumi M, Seki T, Iwasaki K, Sakamoto K. Chinese herbal medicine Yi-Gan-San decrease the lipid accumulation in mouse 3T3-L1 adipocytes by modulating the activities of transcription factors SREBP-1c and FOXO1. Tohoku J Exp Med 2009;219:53-62.

Kim H, Hiraishi A, Tsuchiya K, Sakamoto K. (-) Epigallocatechin gallate suppresses the differentiation of 3T3-L1 preadipocytes through transcription factors FoxO1 and SREBP1c. Cytotechnology 2010;62:245-55.

406 Kim JB, Sarraf P, Wright M, Yao KM, Mueller E, Solanes G, et al. Nutritional and insulin
407 regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. J Clin
408 Invest 1998;101:1–9.
409 Kim JB, Spiegelman BM. ADD1/SREBP1 promotes adipocyte differentiation and gene
410 expression linked to fatty acid metabolism. Genes Dev 1996;10:1096-107.
411 Kim SH, Park HS, Lee MS, Cho YJ, Kim YS, Hwang JT, et al. Vitisin A inhibits adipocyte
412 differentiation through cell cycle arrest in 3T3-L1 cells. Biochem Biophys Res Commun
413 2008;372:108-13.
414 Koutnikova H, Cock TA, Watanabe M, Houten SM, Champy MF, Dierich A, et al.
415 Compensation by the muscle limits the metabolic consequences of lipodystrophy in PPAR
416 gamma hypomorphic mice. Proc Natl Acad Sci USA 2003;100:14457-62.
417 Lee H, Lee YJ, Choi H, Ko EH, Kim JW. Reactive oxygen species facilitate adipocytes
418 differentiation by accelerating mitotic clonal expansion. J Biol Chem 2009;284:10601-9.
419 Lee M-S, Kim C-T, Kim I-H, Kim Y. Inhibitory effect of green tea catechin on the lipid
420 accumulation in 3T3-L1 adipocytes. Phytother Res 2008;23:1088-91.
421 Litridou M, Linssen J, Schols H, Bergmans M, Posthumus M, Tsimidou M, et al. Phenolic
422 compounds in virgin olive oils: fractionation by solid phase extraction and antioxidant activity
423 assessment. J Sci Food Agric 1997;74:169-74.
424 Maeda H, Hosokawa M, Sashima T, Takahashi N, Kawada T, Miyashita K. Fucoxanthin and
425 its metabolite, fucoxanthinol, suppress adipocyte differentiation in 3T3-L1 cells. Int J Mol
426 Med 2006;18:147-52.
427 Manna C, Galletti P, Maisto G, Cucciolla V, D'Angelo S, Zappia V. Transport mechanism
428 and metabolsi of olive oil hydroxytyrosol in Caco-2 cells. FEBS Letters 2000; 470:341-4.
429 Mateos R, Espartero JL, Trujillo M, Rios JJ, Leon-Camacho M, Alcudia F, et al.
430 Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction
431 and high-performance liquid chromatography with diode array ultraviolet detection. J Agric
432 Food Chem 2001;49:2185–92.
433 Rietjens SJ, Bast A, De Vente J, Haenen GRMM. The olive oil antioxidant hydroxytyrosol
434 efficiently protects against the oxidative stress-induced impairement of the NO^{bullet} response
435 of isolated rat aorta. Am J Physiol Heart Circ Physiol 2007; 292:1931-6.
436 Rosen ED, Hsu CH, Wang X, Sakai S, Freeman MW, Gonzalez FJ, et al. C/EBPalpha induces
437 adipogenesis through PPARgamma: a unified pathway. Genes Dev 2002;16:22-6.
438 Ryan D, Antolovich M, Prenzler P, Robards K, Lavee S. Biotransformations of phenolic
439 compounds in *Olea europaea* L. Sci Hortic 2002;92:147-76.

Saltiel AR, Kahn CR. Insulin signaling and the regulation of the glucose and lipid metabolism. Nature 2001;414:799-806.

Santiago-Mora R, Casado-Diaz A, De Castro MD, Quesada-Gomez JM. Oleuropein enhances osteoblastogenesis and inhibits adipogenesis: the effect on differentiation in stem cells derived from bone. Osteoporos Int 2010;22:657-84.

Sekiya M, Hiraishi A, Touyama M, Sakamoto K. Oxidative stress induced lipid accumulation via SREBP1c activation in HepG2 cells. Biochem Biophys Res Commun 2008;375:602-7.

Spiegelman BM, Flier JS. Adipogenesis and obesity: rounding out the big picture. Cell 1996;87:377-89.

Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPARγ, a lipid-activated transcription factor. Cell 1994;79:1147-56.

Trichopoulou A, Costacou T, Bamia C, Trichopoulos D. Adherence to a Mediterranean diet and survival in a Greek population. N Engl J Med 2003;348: 2599-608.

Visioli F, Poli A, Galli C. Antioxidant and other biological activities of phenols from olives and olives oil. Med Res Rev 2002;22:65-75.

Yang JY, Della-Fera MA, Hartzell DL, Nelson-Dooley C, Hausman DB, Baile CA. Esculetin induces apoptosis and inhibits adipogenesis in 3T3-L1 cells. Obesity 2006;14:1691-8.

Figure Legends

Figure 1. Chemical structure of oleuropein and hydroxytyrosol.

Figure 2. Effect of hydroxytyrosol and oleuropein on adipocyte differentiation (A and B). 3T3-L1 cells were harvested and differentiated in the presence of several doses of hydroxytyrosol (0, 50, 100 and 150 μmol/L) (A (a)) or oleuropein (0, 100, 200 and 300 μmol/L) (A (b)) for 8 days. Cells were stained with Oil Red O. Stained intracellular oil droplets were eluted with isopropanol and quantified spectrophotometrically at 420 nm (B). Final concentration of ethanol was 0.1 % for all the samples treated with hydroxytyrosol. Error bars represent a standard error (± SEM); Results are representative of 3 independent experiments with triplicate for each concentration used.

Figure 3. Effect of hydroxytyrosol and oleuropein on 3T3-L1 cell viability (A and B). 3T3-L1 cells were cultured in a 6-cm plate with hydroxytyrosol (0, 100, 150 and 200 μmol/L) (A (a)) or oleuropein (0, 200, 300, 400 μmol/L) (A (b)) for 48 h after confluence. Cells were

trypsinized and their viability was determined by Trypan Blue staining. Preadipocytes were harvested in 24-well plates. After reaching the confluence, cells were treated with (0, 50, 100, 150, 200 μmol/L) hydroxytyrosol (B (a)) or (0, 100, 200, 300, 400 μmol/L) oleuropein (B (b)) for 2 days. Cell viability was identified by the addition of MTT reagent. Final concentration of ethanol was 0.1 % for all the samples treated with hydroxytyrosol. Error bars represent a standard error (± SEM); Results are representative of 3 independent experiments with triplicate for each concentration used in traypan blue assay and sixplicate in MTT assay.

Figure 4. Time course effect of hydroxytyrosol (a) and oleuropein (b) on 3T3-L1 differentiation.

3T3-L1 were differentiated in the presence of several doses of hydroxytyrosol (a) or oleuropein (b) during 0-2 days (early stage), 2-4 days (middle stage), 4-6 days (late stage), and 0-8 days. Total lipid quantity was quantitated via Oil Red O. Final concentration of ethanol was 0.1 % for all the samples treated with hydroxytyrosol. Error bars represent a standard error (± SEM); Results are representative of 3 independent experiments with triplicate for each concentration used.

Figure 5. Effect of hydroxytyrosol and oleuropein on gene expression of PPARγ, C/EBPα (A), CD36 and GLUT4 (B) in 3T3-L1 adipocytes

3T3-L1 cells were cultured 8 days after initiation of differentiation. They were then treated with 0-150 μmol/L of hydroxytyrosol or 0-300 μmol/L of oleuropein for 8 days at 37 °C in a humidified 5% CO_2 incubator. The relative expression of the transcription factor PPARγ and C/EBPα was quantified by qRT-PCR (A). The expression of CD36 and GLUT4 was also quantified by qRT-PCR (B). Final concentration of ethanol was 0.1 % for all the samples treated with hydroxytyrosol. Error bars represent a standard error (± SEM); Results are representative of 2 independent experiments with triplicate for each concentration used.

Figure 6. Effect of hydroxytyrosol and oleuropein on gene expression of SREBP-1c and FASN (A), and the transcriptional activity of SREBP-1c (B)

3T3-L1 cells were cultured 8 days after initiation of differentiation. Cells were treated with 0-150 μmol/L of hydroxytyrosol or 0-300 μmol/L of oleuropein for 8 days at 37 °C in a humidified 5% CO_2 incubator. The relative expression level of SREBP-1c and FASN was quantified by qRT-PCR (A). 3T3-L1 cells transfected by luciferase reporter plasmids that carry a SREBP-1c DNA-binding site (SRE-Luc) were cultured in 6-mm plates in the presence

of hydroxytyrosol (Hd (150 μmol/L)) or oleuropein (Ole (300 μmol/L)) for 48 h. Cells were lysed and luciferase activity was measured (B). Error bars represent a standard error (± SEM), n=3. Final concentration of ethanol was 0.1 % for all the samples treated with hydroxytyrosol. Error bars represent a standard error (± SEM); Results are representative of 2 independent experiments with triplicate for each concentration used.

Figure 7. Effect of hydroxytyrosol (a) and oleuropein (b) on the clonal expansion of 3T3-L1 preadipocytes

Differentiation of 3T3-L1 preadipocytes was initiated in the presence of hydroxytyrosol (0, 100, 150 μmol/L) (a) or oleuropein (0, 200, 300 μmol/L) (b). After 24 h and 48 h, the cells were trypsinized and counted. Error bars represent a standard error (± SEM), n=3. Final concentration of ethanol was 0.1 % for all the samples treated with hydroxytyrosol. Error bars represent a standard error (± SEM); Results are representative of 3 independent experiments with triplicate for each concentration used.

Figure 8. Effect of hydroxytyrosol and oleuropein on cell cycle progress during the mitotic clonal expansion phase (A and B)

3T3-L1 cells were cultured for 24 h after initiation of differentiation in the presence of several doses of hydroxytyrosol or oleuropein. Change of cell cycle was analyzed by flow cytometry (A) and plotted on graph (B). The flow cytometry was performed 2 independent times with duplicate for each concentration used.

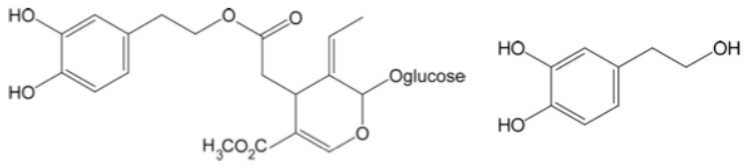
Table 1. Primers for RT-PCR. PCR was performed using the primers indicated as below under optimal amplification condition (95 °C for 5 min; 22-35 cycles of 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; 72 °C for 7 min) for each gene. The PCR amplification of each cDNA was performed independently in triplicate.

Name	Forward	Reverse
G3PDH	5'-GACCCCTTCATTGACCT-3'	5'-CCACCACCCTGTTGCTGT-3'
PPARγ	5'-AAACTCTGGGAGATTCTCCT-3'	5'-TCTTGTGAATGGAATGTCTT-3'
C/EBPα	5'-TCTACGAGGTGGAGCCGC-3'	5'-CCAGCGCCAGCTGCTTCG-3'
GLUT4	5'-TGCTGGGCACAGCTACCC-3'	5'-TATGGCCACGATGGAGAC-3'
CD36	5'-AAACCCAGATGACGTGGC-3'	5'-AGGTCGATTTCAGATCCG-3'
SREBP-1c	5'-TTGTACCCACTGGTAGAGC-3'	5'-CTGTGGCCTCATGTAGGAAT-3'
FASN	5'-GAGCTACCGGGCAAAGAT-3'	5'-AAGGCTCAGTTTGGCTCC-3'

Table 2. Primers for qRT-PCR. PCR was performed using the primers indicated as below under optimal amplification condition (95 °C for 5 min; 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s) for each gene. The PCR amplification of each cDNA was performed independently using three samples in triplicate.

Name	Forward	Reverse
G3PDH	5'-TGGTGAAGGTCGGTGTGAACGG-3'	5'-TGCCGTTGAATTTGCCGTGAGT-3'
PPARγ	5'-AAACTCTGGGAGATTCTCCT-3'	5'-TGGCATCTCTGTGTCAAC-3'
C/EBPα	5'-GCCAAACTGAGACTCTTC-3'	5'-GGAAGCCTAAGTCTTAGC-3'
GLUT4	5'-TGCTGGGCACAGCTACCC-3'	5'-CGGTCAGGCGCTTTAGAC-3'
CD36	5'-AAACCCAGATGACGTGGC-3'	5'-AAGATGGCTCCATTGGGC-3'
SREBP-1c	5'-GCTTAGCCTCTACACCAACTGGC-3'	5'-ACAGACTGGTACGGGCCACAAG-3'
FASN	5'-TGGAGCCTGTGTAGCCTTCGAG-3'	5'-ACAGCCTGGGGTCATCTTTGCC-3'

Fig. 1.



Oleuropein Hydroxytyrosol

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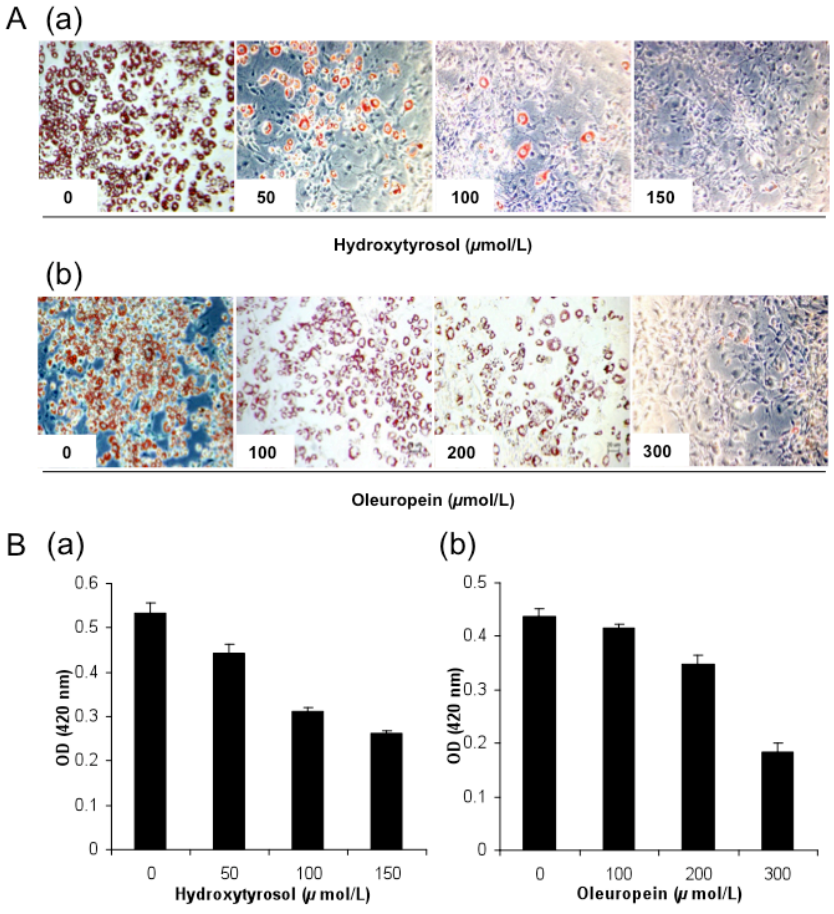
Fig. 2.



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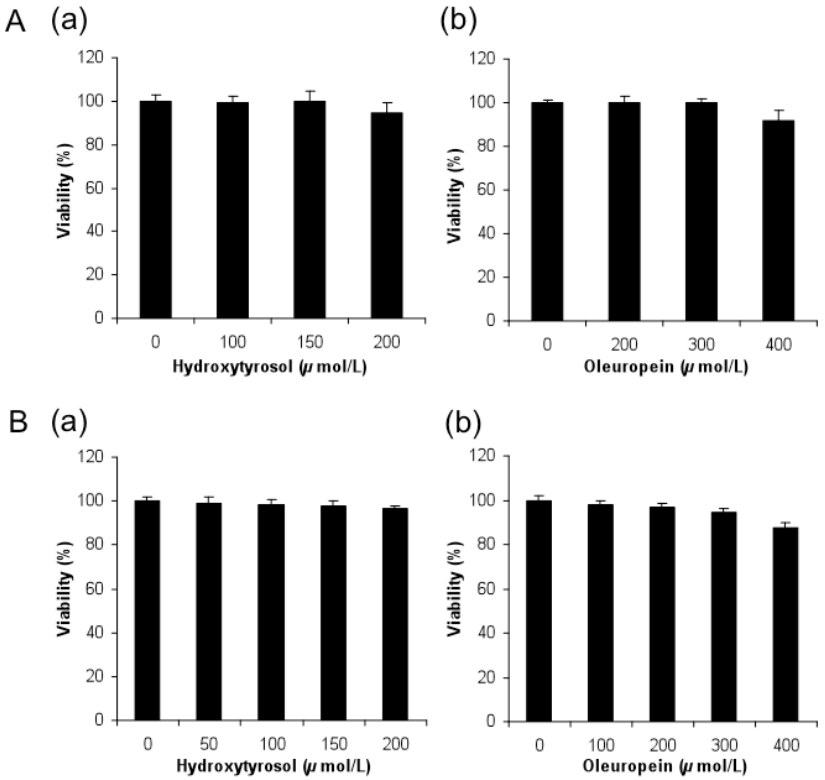

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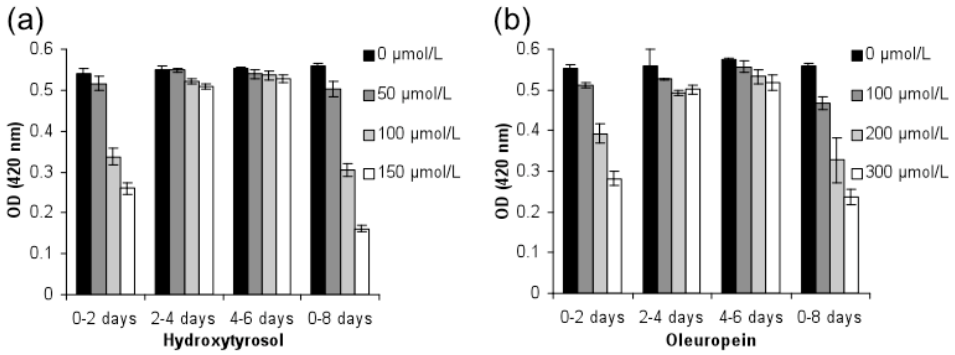

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Fig. 5.

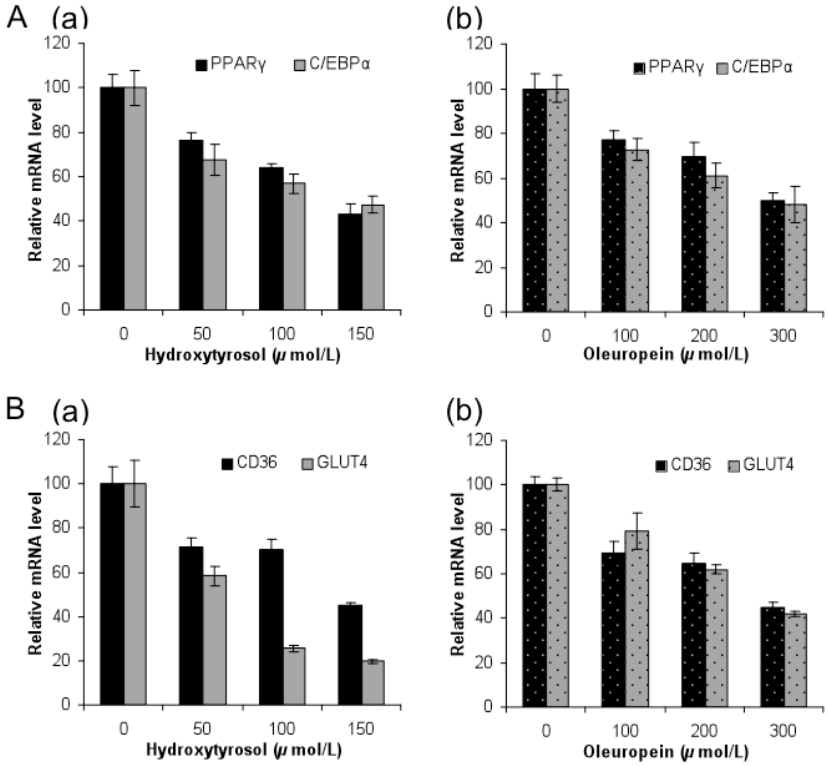

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Fig. 6.

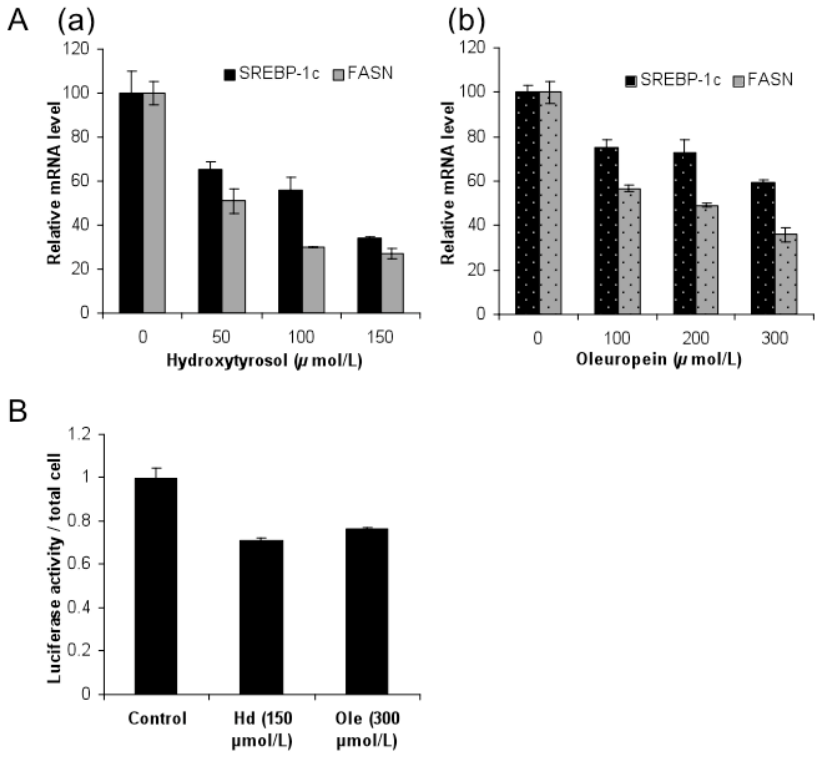

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Fig. 7.

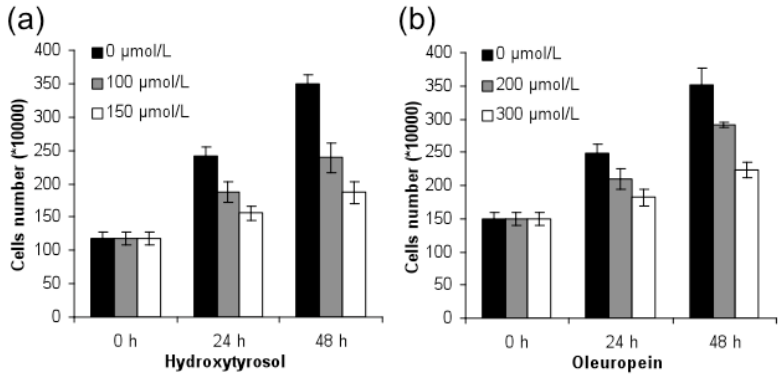

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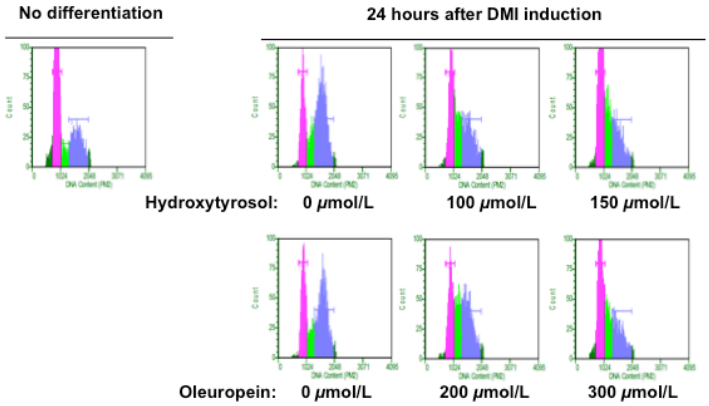
Fig. 8. A



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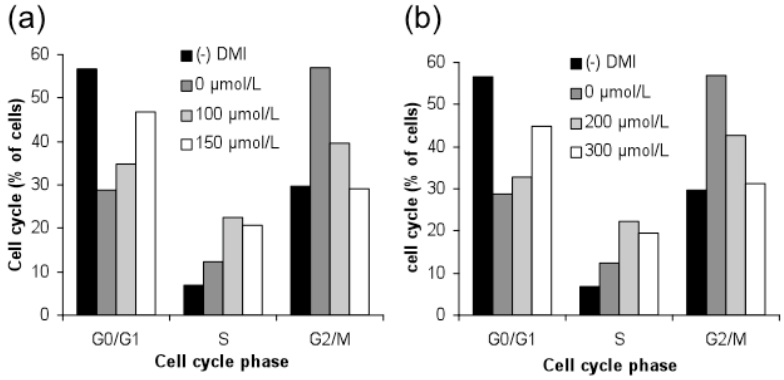

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