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Original / *Otros*

Anti-adipogenic activity of an olive seed extract in mouse fibroblasts

Carmen Veciana-Galindo[1], Ernesto Cortés-Castell[2], Luis Torró-Montell[1], Antonio Palazón-Bru[3], Elia Sirvent-Segura[1], María M. Rizo-Baeza[4] and Vicente F. Gil-Guillén[3]

[1]Laboratorio de Biotecnología y Proyectos, Biopartner S.L., Alcoy. [2]Department of Pharmacology, Pediatrics and Organic Chemistry. Miguel Hernández University. San Juan de Alicante. [3]Department of Clinical Medicine. Miguel Hernández University. San Juan de Alicante. [4]Department of Nursing and Nutrition. University of Alicante. San Vicente del Raspeig, Spain.

Abstract

The administration of different polyphenols protects against increased body weight and fat accumulation. The aim of the study was to determine the anti-adipogenic activity of an olive-seed polyphenolic extract, by means of mouse fibroblast cell line 3T3-L1 adipocyte differentiation.

***Material and methods:* cells were incubated and differentiated (6000 cells/cup) in the presence of olive-seed extract at 10 and 50 mg/l biosecure concentrations of polyphenols, and with no extract in the control sample. After 5 to 7 days mature adipocytes are formed. The fat clusters are quantified by means of red-oil staining, 490 nm absorbance, and the expression of the leptin and PPARg genes, and then compared to the values obtained in the cultures before and after adipocyte differentiation.**

***Results:* the control samples, with no extract, presented an accumulation of fat of 100%. By contrast, the addition of 50 mg/l of olive-seed extract polyphenols resulted in a 50% accumulation of fat, similar to that of the non-differentiated cells. A 10 mg/l extract concentration had no effect. Anti-adipogenic activity is thus confirmed, as the expression of the PPARg and leptin genes is reduced in adipocyte differentiation in the presence of extract at 50 mg/l.**

In conclusion, both the formation of fatty substances characteristic of adipogenesis, and the expression of the adipogenic PPARg and leptin genes are found to be inhibited by the prior addition of olive-seed extract polyphenols at a 50 mg/l concentration.

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Key words: *Adipogenesis. Fat accumulation. Inhibition. Olive-seed extract. Polyphenols.*

ACTIVIDAD ANTI-ADIPOGÉNICA DE UN EXTRACTO DE HUESOS DE ACEITUNA EN FIBROBLASTOS DE RATÓN

Resumen

La administración de diferentes polifenoles protege contra el incremento de peso y la acumulación de grasa.

***Objetivo:* comprobar la actividad anti-adipogénica de un extracto polifenólico de huesos de aceituna, utilizando la diferenciación a adipocitos de la línea celular 3T3-L1 de fibroblastos de ratón.**

***Material y métodos:* se cultivan y diferencian las células (6.000 células/pocillo) en presencia del extracto de huesos de aceitunas a 10 y 50 mg/l de polifenoles, concentraciones bioseguras, y sin extracto como control. A los 5-7 días se forman los adipocitos maduros. Se cuantifican los cúmulos de grasa formados mediante tinción con Oil-Red y medida de la absorbancia a 490 nm y la expresión de los genes de leptina y PPARg, relacionándolos con los valores en los cultivos antes y después de diferenciarse a adipocitos.**

***Resultados:* las muestras control, sin extracto, se consideran el 100% de acumulación de grasas. En contraste, la adición de 50 mg/l de extracto de polifenoles de huesos de aceituna muestra un cúmulo de grasa de alrededor del 50%, semejante a las células no diferenciadas. Con 10 mg/l de extracto no se muestra efecto. Se confirma la actividad antiadipogénica, observándose disminución en la expresión de los genes PPARg y de leptina en la diferenciación a adipocitos en presencia del extracto a 50 mg/l.**

En conclusión, la formación de los cuerpos grasos característicos de la adipogénesis queda inhibida previa adición de 50 mg/l de polifenoles de extracto de huesos de aceituna, así como la expresión de los genes adipogénicos PPARg y de leptina.

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Palabras clave: *Adipogénesis. Inhibición. Polifenoles. Extractos de huesos de aceituna. Cúmulos de grasa.*

Correspondence: Ernesto Cortés Castell.
Profesor. Departamento Farmacología, Pediatría y Química Orgánica. Universidad Miguel Hernández de Elche
E-mail: ernesto.cortes@umh.es

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Introduction

The balance between energy consumption and energy expenditure is very complex, and is influenced by lifestyle, calorie and nutrient intake, energy metabolism, response to stress, immunological metabolism and genes. If this balanced is altered, it could result in obesity. Fat metabolism is a significant indicator of this balance, with peroxisome proliferator-activated receptors (PPAR), leptin and adiponectin gene expression[1].

It has been demonstrated that polyphenols have a beneficial effect on the adipogenesis in obese patients. Obesity has a strong inflammatory component which can potentially be affected by diet, and polyphenols can form part of this anti-inflammatory nutritional response[2-4]. Furthermore, there are also studies which demonstrate the effect of polyphenols in reducing fat deposits in rats[5].

Dietary supplements containing polyphenol compounds are associated with a reduction of diet-induced obesity[6,7]. Recent studies indicate that these anti-obesity effects come about through the genes involved in adipogenesis, lipolysis and the oxidation of fatty acids[8].

Due to the powerful antioxidant activity and anti-inflammatory properties of polyphenols derived from the olive, they are the object of numerous studies[9-11]. Likewise, very recently olives, and the fractions deriving from olive production, have been linked to their effects on obesity-related mechanisms[12-13].

In our literature research we found no papers related to the anti-adipogenic effect of extracts with olive-seed polyphenols, although our research group showed the anti-inflammatory effect of this extract[14]. Therefore, we carried out a study assessing this issue. To this end, the cell line 3T3-L1 model was used, which, together with the right inducers (insulin, dexamethasone, and IBMX) can result in adipocyte differentiation, and as an assay biomarker a characteristic feature of adipogenesis was used: namely, the formation of body fat and the expression of the genes PPARγ, and leptin, which favour adipogenesis.

Material and methods

Assessment of the regulatory activity of the fatty tissue was carried out by means of the cellular line of mouse fibroblasts which are capable of 3T3-L1 (CL-173 [ATCC]) adipocyte differentiation, and are therefore a good model for obesity-related studies[15].

For the assay, cells 3T3-L1 (CL-173 [ATCC]) were differentiated in the presence of a 10 and 50 mg/l olive seed extract, concentrations which had previously been shown to be biosecure[16], with the same cultures being used as a control sample, but without the addition of the extract.

The assay was performed on 96 cup thermal cycler plates, with an initial seeding of 6,000 cells/cup. With two days incubation at 37 ºC and 5% CO_2 in the fibroblast growth medium DMEM: 4% glucose, 10% CS, 2mM de L-glutamine and Penicillin (100 U/ml)-Streptomycin (10 g/ml). Differentiation is initiated by substituting the growth medium for the differentiation 1 medium (DMEM/10% fetal calf serum/0.5 mM IBMX/1 μM dexamethasone). After two days the medium was replaced with the differentiation 2 medium (DMEM/10% fetal calf serum/+10 μg/ml insulin). After three days, the medium was replaced with the maintenance medium (DMEM/10% fetal calf serum). The adipocyte is considered mature after 5-7 days of differentiation[17,18]. The differentiation process involves molecular and metabolic changes, as well as morphological changes.

Screening of the bioactive extract responsible for modulating obesity was performed by means of “high-throughput screening”, using as an obesity biomarker the analysis of the capacity to inhibit the formation of accumulation of fat during adipogenesis[19,20]. Thus, once the differentiation process was completed, the formation of fat accumulation was analysed by means of a colourimetric technique with Oil-Red staining (International Chemical). This compound stains the accumulated fats, which are quantified by measuring A_{490nm}. The resulting accumulation of fat is directly proportional to A_{490nm}, so that the changes in absorption are related to the changes in lipid concentration. The expression of the results is carried out with reference to the control cells, cells which have been differentiated in a similar fashion, but with no extract being added, on the basis of the following formula:

% Lipids = (A_{490nm} sample / A_{490nm} control cells differentiated) x 100.

For the analysis of the gene expression, RNA was extracted from the cultured and differentiated cells, without and with extract (50 mg/l of polyphenols). The extraction is carried out with the kit Rneasy Mini Kit (74104 Quiagen). From the RNA, c-DNA was obtained by means of the transcriptase enzyme (High Capacity cDNA reverse transcription kit, 4368814, Applied Biosystems), and with the cDNA obtained, a real-time PCR was performed for the PPARγ and leptin genes, with the Mm00440181_m1 primers (Applied Biosystems) for the leptin, and Mm01184322_m1 (Applied Biosystems) for the PPARγ, the control gene being GADPH Mm99999915_g1 (Applied Biosystems). Reaction conditions were 10μl (1x) TaqMan Gene Expression Mastermix 4369016 Applied Biosystems; 2μl (0.3μM) for each primer, 2μl (0.1μM) for the marker gene sonda, 0.4μl for the PCR internal reaction control, (AND exo 50x), 2μl Mix EXO y 2μl (50ng) de c-ADN. The amplification conditions in the thermocycler (7300 Applied Biosystem) were universal. 50 ºC during 2 min, 95 ºC during 10 min and 40 cycles of: 95 ºC - 15 sec and 60 ºC - 1 min. Gene expression was quantified relatively, the physiological changes in the biomarker genes being measured by comparison with the reference gene.

All the assays were carried out during two different periods, and in each case they were duplicated (n = 4). The data was analysed statistically by means (t-test) of the Epidat 3.1 programme.

Results

The adipocyte cells were round and present an accumulation of fatty substances (Fig. 1). This figure shows the formation of adipocytes which have been produced in the culture and differentiation cups. Before proceeding to analyse the accumulation of fatty tissue and the gene expression, the cellular viability of line 3T3-L1 was examined following the adipogenesis process in the presence of the extract at maximum concentration of 50 mg/l polyphenols, and a viability of 100.4% (SD 2.3; 95% CI: 96.8-104.0%) was observed.

The adipocytes present a rounded shape and the internal accumulation of fatty substances.

In the culture cups, the fatty substances were quantified in differentiated cells 3T3-L1 in the presence of the extract, at concentrations of 10 mg/l and 50 mg/l, and only with the culture media as a control. The results are expressed in table I, in the form of comparative data obtained with reference to the differentiated control cells (without the extract), with the result that 100% of these differentiated cells are shown to present accumulation of fatty tissue, and in comparison with this value, the cells treated with the samples show the presence of lipids. It must be pointed out that the cells before adipocyte differentiation present 57.9% (SD 5.3; 95% CI: 49.5-66.3%) of accumulated fat with respect to the differentiated ones, and that only a 50 mg/l concentration shows significant anti-adipogenic activity (p=0.021), with relative fatty tissue values of 49.5% (SD 4.4; 95% CI: 49.5-56.5%).

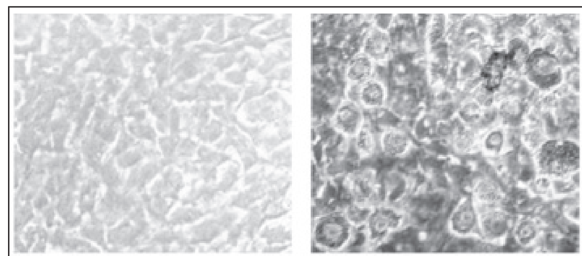

Fig. 1.—Effect of adipogenesis on cell line 3T3-L1 after 5 days incubation with differentiation media (2 days with differentiation medium 1 (DMEM/10% fetal calf serum/0.5 mM IBMX/1 µM dexamethasone, followed by 3 days with differentiation medium 2 (DMEM+10% fetal calf serum+10 µg/ml insulin).
Left part: Cell line 3T3-L1preadipcytic state (undifferentiated), presents fibroblast morphology in culture (striated and elongated cell).
Right part: Cell line 3T3-L1 after adopgenesis.

Anti-adipogenic activity, based on the capacity of olive seed extract at 50 mg/l to inhibit the formation of fatty substances during adipocyte differentiation, deals with practically all the accumulation of fat, leaving the cells differentiated in the presence of the extract with similar amounts of fatty tissue to those present in the cells prior to differentiation.

On the basis of the results obtained and represented, the addition of olive seed extract at 50 mg/l of polyphenols was observed to be extremely effective in inhibiting the formation of fatty substances during adipogenesis.

Table II shows the results of the gene expression of the leptin and PPARγ genes in cells 3T3-L1 before and after the process of adipogenesis, in the absence (control group) and in the presence of the extract. Normalised values in the undifferentiated cells are presented by means of the formula 2-ΔΔCt (GAPDH was used as an internal control gene). In this table it can be observed that the expression of the leptin gene increases significantly (p=0.014) during the differentiation from 0.05 (95% CI: 0.03-0.07) to 1.1 (95% CI: 0.5-1.7), with the said expression being inhibited some 5 times, until it reached 0.2 (95% CI: 0.1-0.3) significantly (p=0.022) when the differentiation was performed in the presence of the extract at 50 mg/l. Similar results were observed for gene PPARγ, which during differentiation changes from 0.5 (95% CI: 0.4-0.7) to 1.5 (95% CI: 0.8-2.3) with significant differences (p=0.030), and in the presence of the extract to 1.0 (95% CI: 0.5-1.5).

Discussion

Adipocyte dysfunction is closely linked to the development of obesity and resistance to insulin. The

Table I

Evaluation of fat accumulation in 3T3-L1 cells prior to and following the adipogenesis process (adipocyte differentiation) in the presence of olive extract. The values relate to the differentiated cells with 100% fatty substance deposits

Study groups	*% Fat mean±SD (95% CI)*	*p-value for the means difference with differentiated cells*
Differentiated cells	100.0 ± 22.2 (64.7-135.3)	N/A
Undifferentiated cells	57.9 ± 5.3 (49.5-66.3)	0.034
Differentiated cells + 10 mg/l extract	109.3 ± 11.8 (90.5-128.1)	0.487
Differentiated cells + 50 mg/l extract	49.5 ± 4.4 (42.5-56.5)	0.021

Abbreviations: CI, confidence interval; N/A, not applicable; SD, standard deviation.

Table II
Gene expression of the leptin and PPARγ genes in 3T3-L1 cells prior to and following the process of adipogenesis, in the absence (control) and presence of 50 mg/l olive seed polyphenol extract

Study groups	*Leptin gene Mean±SD (95% CI)*[a]	*PPARγ gene Mean±SD (95% CI)*[b]
Undifferentiated cells	0.05 ± 0.01 (0.03-0.07)	0.5 ± 0.1 (0.4-0.7)
Differentiated cells	1.1 ± 0.4 (0.5-1.7)	1.5 ± 0.5 (0.8-2.3)
Differentiated cells with 50 mg/l extract	0.2 ± 0.1 (0.1-0.3)	1.0 ± 0.3 (0.5-1.5)

[a]Means difference between undifferentiated cells and differentiated cells: p=0.014; means difference between undifferentiated cells and differentiated cells with 50 mg/l extract: p=0.059; means difference between differentiated cells and differentiated cells with 50 mg/l extract: p=0.059.
[b]Means difference between undifferentiated cells and differentiated cells: p=0.030; means difference between undifferentiated cells and differentiated cells with 50 mg/l extract: p=0.020; means difference between differentiated cells and differentiated cells with 50 mg/l extract: p=0.137.

adipocytes synthesise and secrete active molecules known as adipokines (such as leptin for example), which reduces the absorption of food and increases energy expenditure.

In this study we used the 3T3L1 cell line of mouse fibroblasts (pre-adipocytes) from the American Type Culture Collection (ATCC), obtained from a continuous 3T3 extract, and characterised by the fact that it is made up of unipotent cells which have the ability to express insulin receptors, and which have been used extensively as models for the study of adipogenesis[20]. The lipogenesis process in the adipocytes was evaluated in terms of the number of triglycerides present in the fatty cells, by means of a quali-quantitative method consisting of staining the cells with red oil, which results in the triglycerides being colored[21].

The regulatory activity of the fatty tissue was evaluated via a fibroblast cell-line model capable of adipocyte 3T3-L1 (CL-173 [ATCC]) differentiation. The process of the formation and development of the adipocyte was also evaluated via an analysis of the genes that participate in the process: the PPARγ gene (a transcriptional factor which in turn modulates the expression of other genes) and the leptin gene. Both genes were expressed during adipogenesis. The fatty tissue is considered to have a regulatory effect if it modifies the expression of the genes, and anti-obesity activity is demonstrated if the expression of the marker genes diminishes[1,20,22,23].

Studies dealing with the effects of polyphenols and flavonoids on adipocytes are much less frequent than in relation to any other type of cell, but some very interesting results are coming to light. Quercetin and fisetin have been shown to enhance epinephrine-induced lipolysis in isolated adipocytes in rats, and seem to increase the methylation of the phospholipids in the membrane associated with cyclic AMP cell accumulation[24]. It has been discovered that quercetin blocks insulin-dependent lipogenesis, thus inhibiting the activity of insulin tyrosine kinase from substrate-level phosphorylation.

These lipocyte and anti-polygenic effects in rat adipocytes go hand in hand with the antipoliferative activity in one cell-type, which suggests that flavonoids can reduce the mass of fatty tissue, thus inhibiting the signals which promote adipogenesis[25]. By using 3T3-L1 cells it can be shown that genistein and naringenin inhibit the proliferation of preconfluent pre-adipocyte cells, but only genistein was able to inhibit post-confluent proliferation in differentiation induction and subsequent mature adipocyte differentiation. In mature adipocytes, it was shown to be highly effective in inducing lipolysis, both on its own and in combination with epinephrine[26]. These discoveries in adipose cells suggest that the presence of flavonoids in the diet, in particular genistein, can have inhibitory effects on the growth of fatty tissue. Genistein offers promising prospects for the nutrient-mediated regulation of body fat due to its effects on preadipocyte replication, differentiation and lipolysis. Genetic expression studies, in particular those which use adipocyte RNA microarrays, have brought to light some interesting additional polyphenol effects.

Other authors[27,28] have shown that grape-seed and skin extracts are richs in polyphenol compounds, and that these compounds inhibit the enzymes which metabolise fat, pancreatic lipase and hormone-sensitive lipase. Consequently, the authors described this effect as a possible approach to weight-control which would be safe, natural and cost-effective.

Resveratrol has shown Anti-adipogenic activity in mesenchymal cells differentiation to adipocytes, a hypolipidemic effect, and PPAR and lipoprotein lipase genes expression reduction. This effect can be synergic with other fitochemical products[28,29].

Flavonoid-type polyphenols have also been shown to inhibit glucose transport. The regulation of glucose absorption, under the control of insulin, is crucial for maintaining adequate glucose levels in the blood during food-intake and fasting.

In addition, the polyphenols of Rosmarinus officinalis (rosemary) have been effective in preadipocyte 3T3-L1 differentiation to inhibit the accumulation of triglycerides[30]. The extract inhibited adipocyte differentiation and the molecular events taking place during the process of adipogenesis involve early (C/EBPβ) and late (PPARγ) response transcription factors, the results of which show that the polyphenols and components of rosemary extract have an anti-adipogenesis effect in that they play a part in the expression of transcription factors that are crucial to the acquisition of the adipocytic phenotype, which has a beneficial effect in

the treatment of obesity and related illnesses. Furthermore, the green tea increases the fatty oxidation and the thermogenic effect[31].

Conclusion

The present study has focused on the inhibition of the formation of the fatty substances characteristic of adipognesis, through the use of an olive seed extract rich in oleuropeine and hydroxytyrosol, the conclusion being drawn through a quantitative analysis that extract concentrations of 50 mg/l are capable of preventing the complete formation of fatty substances. This inhibition of adipognesis is confirmed by the reduction in the expression of the leptin and PPARγ genes.

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