Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 961–967
Journal of Nutritional Biochemistry

Effects of a grapeseed procyanidin extract (GSPE) on insulin resistance☆

Gemma Montagut, Cinta Bladé, Mayte Blay, Juan Fernández-Larrea, Gerard Pujadas, M. Josepa Salvadó, Lluís Arola, Montserrat Pinent, Anna Ardévol*

Department of Biochemistry and Biotechnology, Rovira i Virgili University, Tarragona, 43007 Spain

Received 18 February 2009; received in revised form 31 July 2009; accepted 18 August 2009

Abstract

Flavonoids are beneficial compounds against risk factors for metabolic syndrome, but their effects and the mechanisms on glucose homeostasis modulation are not well defined. In the present study, we first checked the efficacy of grapeseed procyanidin extract (GSPE) for stimulating glucose uptake in insulin-resistant 3T3-L1 adipocytes. Results show that when resistance is induced with chronic insulin treatment, GSPE maintain a higher stimulating capacity than insulin. In contrast, when dexamethasone is used as the resistance-inducing agent, GSPE is less effective. Next we evaluated how effective different GSPE treatments are at improving glucose metabolism in hyperinsulinemic animals (fed a cafeteria diet). GSPE reduced plasma insulin levels. The lower dose (25 mg GSPE/kg body weight per day) administered for 30 days improved the HOmeostasis Model Assessment-insulin resistance index. This was accompanied by down-regulation of Pparg2, Glut4 and Irs1 in mesenteric white adipose tissue. Similarly, a chronic GSPE treatment of insulin-resistant 3T3-L1 adipocytes down-regulated the mRNA levels of those adipocyte markers, although cells were still able to respond to the acute stimulation of glucose uptake.

In summary, 25 mg/kg body weight per day of GSPE has a positive long-term effect on glucose homeostasis, and GSPE could be targeted at adipose tissue, where it might directly stimulate glucose uptake. This work also highlights the need to carefully consider the bioactive dose, since a higher dose does not necessarily correlate to a greater positive effect.

Keywords: High-fat diet; Adipose tissue; Obesity; Procyanidins; Insulin resistance

1. Introduction

Metabolic syndrome and its association with an increased risk of cardiovascular disease and Type 2 diabetes is an important public health concern. First-line therapy for managing the metabolic syndrome is to modify the patient's lifestyle. For this reason, the Mediterranean diet, rich in flavonoids, can be considered an effective treatment for improving health in patients with metabolic syndrome [1–3]. Of all the ways in which flavonoids act on risk factors, the way they modulate glucose homeostasis is the least well defined. Some studies on humans have shown positive flavonoid effects. Taking 30 mg/day of polyphenol for 18 weeks tended to decrease glycemia in normoglycemic individuals [4]. Ingesting green tea for 32 weeks initially improved several blood parameters (e.g., glucose), though this was reversed by day 87 [5]. In fact, results on humans are controversial, partly due to the diversity of the phenolic structures assayed, that is, flavonoids include hundreds of monomeric structures, oligomers, polymers and modified forms.

Several studies on rodents have shown that monomeric flavonoids are antihyperglycemic agents in some models of insulin resistance (IR) [6–11]. Also, extracts containing a mixture of flavonoid structures can have antihyperglycemic effects. In fact, food contains mixtures of flavonoid structures and different degrees of polymerization, and this makes it difficult to establish which of the molecules the active one is. Furthermore, it is likely that the effects are not due to one molecule but to synergic effects [12]. Up to now, most work has been done in streptozotocin (STZ)-diabetic rats [13–16]. However, STZ-animals are not an IR model. There has been much less work done on insulin resistant models. Al-Awwadi et al. [17] have proven that an extract rich in grape oligomeric procyanidins restores glucose levels in a fructose-induced resistance model, and Lee et al. [18] also proved the positive effects of Persimmon peel procyanidins on db/db mice.

The mechanisms that exert such effects might vary among flavonoids, though many of the studies on monomerics flavonoids found increased glucokinase (Gck) mRNA expression in liver, highlighting this gene expression as a target for antihyperglycemic monomeric flavonoid effects [7–9,19]. Most studies on procyanidin extracts mainly rely on their analysis of antioxidant or anti-inflamatory effects [17,18].

☆ This study was supported by grant numbers AGL2008-01310/ALI, AGL2008-00387/ALI from the Ministerio de Ciencia e Innovacion (MICINN) of the Spanish Government. Gemma Montagut is a recipient of a fellowship from the Rovira i Virgili University in Tarragona.

* Corresponding author. Departament de Bioquímica i Biotecnologia, C. Marcel.lí Domingo, s/n, 43007, Tarragona, Spain. Tel.: +34 977 559566; fax: +34 977 558232.

E-mail address: anna.ardevol@urv.cat (A. Ardévol).

0955-2863/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2009.08.001

In this paper, we analyze how effectively a grape seed procyanidin extract ameliorates several insulin-resistant situations. Such experimental conditions would be of great importance in the field of functional food design, where the effects of natural compounds must be evaluated in a context of middle/long-term administration, and carefully considering the doses that might be administered. In fact, our results highlight the importance of cautiously interpreting results obtained from only one flavonoid-treatment condition.

2. Materials and methods

2.1. Cell culture and glucose uptake assay

3T3-L1 preadipocytes were cultured and induced to differentiate as previously described [20]. Insulin resistance was induced in mature adipocytes by using different agents: 1 nM insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark) for 15 h, 3 nM tumor necrosis factor–α (TNF-α) (ProSpec-Tany TechnoGene, Rehovot, Israel) for 5 h, 25 mU/ml glucose oxidase (Sigma, Madrid, Spain) for 2 h on depletion media (serum-free supplemented Dulbecco's Modified Eagle Medium from containing 0.2% bovine serum albumin) and 100 nM dexamethasone (Sigma) for 24 h. Phosphate-buffered saline solution was used as control. During the last 30 min of incubation, adipocytes were treated with either 100 nM insulin or 100 mg/L grapeseed procyanidin extract (GSPE), and then glucose uptake was assayed. GSPE concentration assayed was nontoxic for 3T3-L1 adipocytes [20,21]. The effects of chronic GSPE treatment in insulin-induced IR were analyzed by preincubating 3T3-L1 adipocytes for 2 h with 100 mg/L GSPE and maintaining them for a further 15 h with 1 nM insulin. GSPE or insulin were acutely stimulated for 30 min in these long-term treated cells, and glucose uptake was analyzed. Glucose uptake was determined by measuring the uptake of 2-deoxy-D-[3*H*] glucose as previously described [16]. Each condition was run in triplicate. According to the manufacturer (DRT, Dax, France), GSPE essentially contains the following procyanidins: monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units; 31.7%).

2.2. Animal experimental procedures

Wistar female rats weighting between 160 and 175 g were purchased from Charles River Laboratories (Barcelona, Spain) and housed in animal quarters at 22°C with a 12-h light, 12-h dark cycle. After 1 week in quarantine, the animals were divided in two groups: a control group (12 animals) fed with a standard diet (Panlab A03) and a cafeteria group (36 animals) fed with a cafeteria diet (bacon, sweets, biscuits with pâté, cheese, muffins, carrots, milk with sugar) and water plus the standard diet. Animals were fed ad libitum, and the food was renewed daily.

Obesity was induced in the animals with cafeteria diet for 13 weeks. Afterwards, the cafeteria group was divided in three subgroups (12 animals/group): (i) cafeteria group: rats treated with a vehicle (sweetened condensed milk), (ii) cafeteria+25: rats treated with 25 mg of GSPE/kg of body weight (bw) per day and (iii) cafeteria+50: rats treated with 50 mg of GSPE/kg of bw per day. The lower GSPE dose was calculated on the basis of a daily moderate human consumption of 0.5 L wine (containing 3 g/L of total polyphenols), that is, 21.42 mg/kg of polyphenols. Rats were fed either the vehicle or GSPE dissolved in the vehicle by controlled oral intake with a syringe. After 10 days of GSPE treatment, six animals from each group were sacrificed (short treatment). After 30 days of GSPE treatment, the remaining six animals of each group were sacrificed (long treatment). Schematic diagram of procyanidin treatments is shown in Scheme 1. Three days before the sacrifice, an intraperitoneal glucose tolerance test (IPGTT) was carried out (2 g glucose/kg bw). Glucose was measured with a glucometer after blood samples had been collected by tail bleeding (Glucocard, Menarini, Barcelona, Spain). The degree of IR was estimated at the baseline by HOmeostasis Model Assessment (HOMA) computed with the formula plasma glucose (mmol/L) × serum insulin (μU/ml)/22.5 according to the method described by Matthews et al. [22].

At 9 a.m. on sacrifice day, the rats were treated with GSPE or vehicle and food was renewed. At 11 a.m. food was withdrawn. After 3 h of fasting the animals were sacrificed by beheading. Blood was collected using heparin and animal tissues were excised, frozen immediately in liquid nitrogen and stored at −80°C until analysis. All the procedures were approved by the Experimental Animals Ethics Committee of the Rovira i Virgili University.

Glucose plasma levels were assayed using an enzymatic colorimetric kit (GOD-PAP method from QCA, Tarragona, Spain). Hormone plasma levels were determined using ELISA kits following the manufacturers' instructions (Rat insulin from Mercodia, Uppsala, Sweden, and leptin from LINCO, Millipore Iberica s.A.U., Madrid, Spain). Measurements were taken with an ELISA Anthos Zenyth 200 st Microplate Reader.

2.3. Quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from adipocytes grown in 6-well plates using the High Pure RNA Isolation Kit (Roche). mRNA levels were measured using real-time reverse transcriptase-polymerase chain reaction as previously described [23]. Amplifications were performed using the following primers, Glut4 CAACGTGGCTGGGTAGGCA (forward) and ACACATCAGCCCAGCCGGT (reverse); Irs1 CCCACAGCAGATCATTAACC (forward) and AGAGACGAAGATGCTGGTGC (reverse); adiponectin GATGGAGGAGCA-CAGAGCC (forward) and GGCCGTTCTCTTCACCTACG (reverse); resistin GTACCCACGG-GATGAAGAACC (forward) and GCAGACCCACAGGAGCAG (reverse); Pparg2 CTGTTGACCCAGAGCATGGT (forward) and AGAGGTCCACAGAGCTGATTCC (reverse); C/EBPα GGTGGACAAGAACAGCAACGA (forward) and CGTTGCGTTGTTTGGCTTTATC (reverse). The mRNA levels of each gene were normalized to the level of glyceraldehyde-3-phosphate dehydrogenase mRNA detected in each sample [CATGGCCTTCCGTGTTCCT (forward) and CCTGCTTCACCACCTTCTTGA (reverse)].

Total RNA from liver and adipose tissue were extracted using TRYZOL reactive following the manufacturer's instructions. cDNA was generated using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Quantitative polymerase chain reaction (PCR) amplification and detection were performed by using specific TaqMan Assay-On-Demand probes (Applied Biosystems, Rn00562597-m1 for Glut4, Rn01495763-g1 for Srebp1c, Rn00440945_m1 for Pparg2, Rn02132493-s1 for Irs1, Rn00565347_m1 for Glucose-6-phosphatase (G6p) and Rn00561265_m1 for Gck). Cyclophiline was used as the reference gene in quantitative PCR (Applied Biosystems TaqMan Assay-On-Demand probe Rn00690933_m1). Reactions were run on a quantitative Real-Time PCR 7300 System (Applied Biosystem) according to the manufacturer's instructions. Quantifications were performed in triplicate.

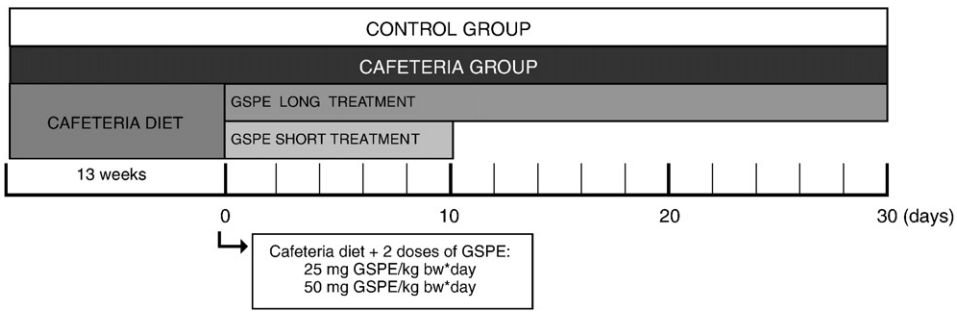
2.4. Calculations and statistical analysis

Results are expressed as the mean±S.E.M. Effects were assessed using one-way analysis of variance or Student's *t* test. All calculations were performed using SPSS software (SPSS, Chicago, IL, USA).

3. Results

3.1. GSPE acutely stimulate glucose uptake better than insulin does in chronic insulin-induced IR in 3T3-L1 adipocytes

Previous results led us to postulate that GSPE could be effective as a preventive and/or corrective agent against IR. We now evaluated this role in 3T3-L1 adipocytes under different metabolic insults. We selected four well-validated experimentally models of IR, all of them with physiological relevance in vivo and involved in different cellular response pathways: chronic hyperinsulinemia [24], TNF-α [25], dexamethasone [26] and oxidative stress treatment [27].



Scheme 1. Schematic diagram of procyanidin treatments.

Fig. 1 shows the stimulation of glucose uptake by acute insulin or GSPE treatment in 3T3-L1 cells preincubated with the IR-inducing agents. Fifteen h of 1 nM insulin treatment increases the basal glucose uptake (results not shown), but limits maximal acute insulin stimulation to 23.6%. Under this resistant situation, an acute stimulation with GSPE (100 mg/L) reaches 47.2% of its maximal stimulation under a nonresistant situation (Fig. 1A). GSPE was not observed to be more efficient, at acutely stimulating glucose uptake, after any of the other IR conditions. After inducing IR by treating the 3T3-L1 cells with glucose oxidase 25 mU/ml (Fig. 1C), insulin acute stimulation reached 70.6% of its maximal effect. Acute GSPE treatment leads to similar results. Following 5 h of 3 nM TNF-α treatment (Fig. 1B), an acute dose of insulin only increases glucose uptake to 57.4% of maximal possible stimulation. Similarly GSPE stimulates 49.1% of its maximal acute effect. However, after 24 h of 100 nM dexamethasone treatment (Fig. 1D), acute GSPE was less efficient at stimulating glucose uptake, since it only reached 59.7% of its maximal possible effect without resistance, while acute insulin showed 82.5% of its effect without resistance.

3.2. Long-term GSPE effects on insulin-induced IR in 3T3-L1 cells

We next checked whether a long-term treatment with GSPE improved chronic insulin-induced resistance in 3T3-L1 adipocytes. Fig. 2C shows that a long-term GSPE treatment starting 2 h previous to chronic (15 h) insulin treatment and maintained until the end of IR induction tended to slightly improve the acute (30 min) insulin and GSPE stimulation of glucose uptake. Next, we evaluated the effects of the long-term GSPE treatment on the mRNA levels of some key glucose uptake proteins, insulin signalling, adipokines and differentiation markers. Surprisingly, we found that this long-term (17.5 h) GSPE treatment caused a strong down-regulation of Glut4, Irs1, adiponectin, resistin, Pparg2 and Cebpa (Table 1), despite the cells maintaining their capacity to acutely respond to insulin and GSPE (shown in Fig. 2C).

3.3. GSPE reduces insulinemia in cafeteria fed rats

We tested in vivo the effects of GSPE in hyperinsulinemic animals. We used a mild model of IR, provoked by feeding healthy female Wistar rats with a cafeteria diet for 13 weeks. Then we started four different GSPE treatments. Table 2 summarizes the chronic observed effects of these treatments. GSPE did not modify the increase in body weight induced by the cafeteria diet, and it did not change plasma leptin levels either. Only 25 mg of GSPE/kg bw per day for 30 days significantly reduced the total amount of visceral adipose tissue. A main feature of GSPE treatment was to reduce the fasting plasma insulin levels, the 25 mg of GSPE/kg bw per day dose being the most effective. GSPE at this dose reduced insulinemia both after 10 and 30 days. However, only the animals receiving the longer dose maintained their fasting glycemia and showed a healthier HOMA-IR index. In the 30-day-treated animals, an IPGTT of 2 h showed lower insulinemia with the same profile, although there was no improvement in glycemia (Fig. 3). We also analyzed the insulin and glucose levels of these animals on the day of sacrifice. Because we sacrificed them 5 h after the last GSPE treatment, they reflect the acute GSPE effects after 30 days of treatment. Table 3 shows that insulinemia was still lower than in cafeteria group, but in this case, glycemia was also reduced by GSPE compared to cafeteria effects.

3.4. White adipose tissue is very sensitive to GSPE treatments

Finally, we analyzed the modulation of gene expression in the different animal groups on the day of sacrifice. Fig. 4A shows that in white adipose tissue (WAT), the cafeteria diet down-regulated mainly genes involved in initial insulin signaling and insulin-depending glucose uptake (according to the peripheral IR suggested by plasma

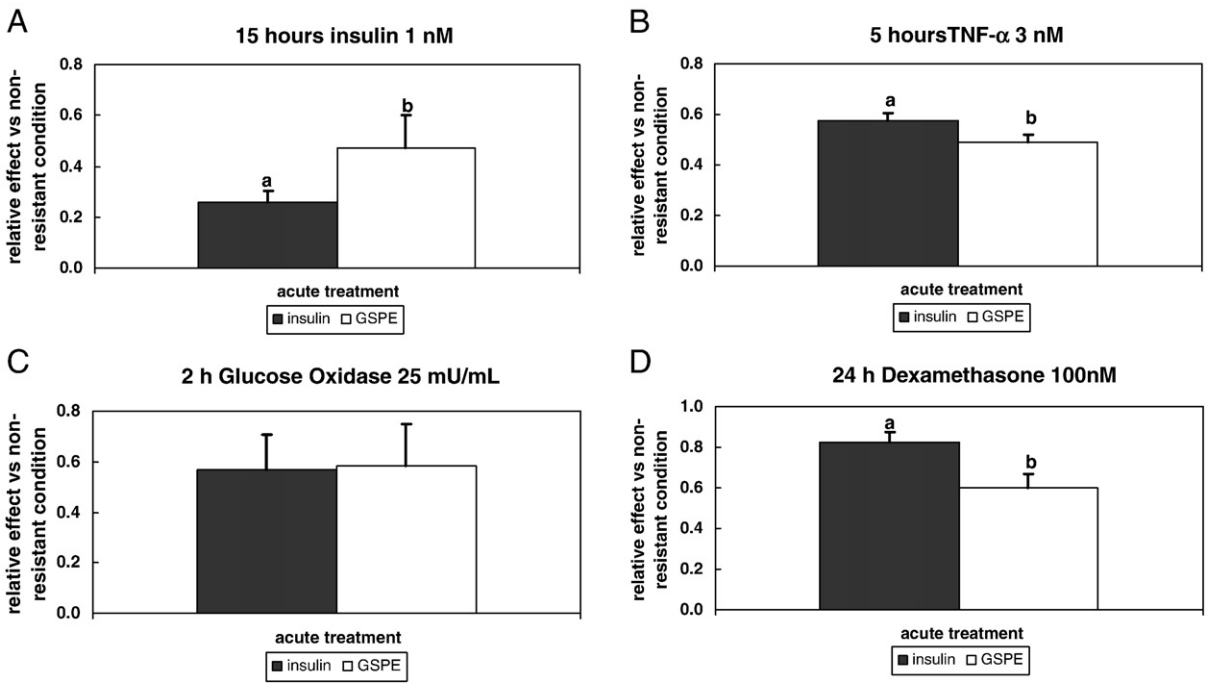


Fig. 1. Effects of acute GSPE treatment on glucose uptake in insulin resistant 3T3-L1 adipocytes. Fully differentiated adipocytes were incubated with four different IR-inducing agents: (A) 1 nM insulin for 15 h; (B) 3 nM TNFα for 5 h (C) 2 h depletion media+2 h 25 mU/ml glucose oxidase on depletion media and (D) 100 nM dexamethasone for 24 h. During the last 30 min of incubation, adipocytes were treated with either 100 nM insulin or 100 mg/L GSPE and then 2-deoxy-d-[^{3}H] glucose uptake was measured. Values represented are, respectively, the insulin or GSPE-induced acute stimulation effects after IR was induced. Each value represents the mean±S.E.M. at least of two experiments run in triplicate. Different letters indicate statistically significant differences between treatments ($P<.05$); for Fig. 1A $P<.1$.

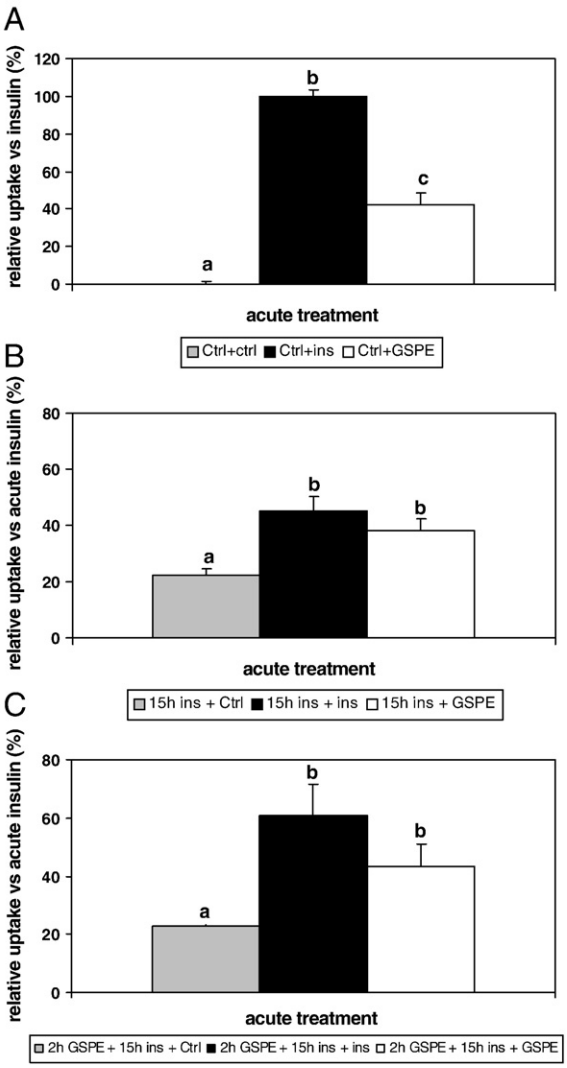


Fig. 2. Effects of a long-term treatment of GSPE on insulin or GSPE-induced glucose uptake stimulation in resistant 3T3-L1 adipocytes. a: controls without IR induction; b: fully differentiated 3T3-L1 adipocytes were incubated for 15 h with 1nM insulin in order to induce IR; c: prior to the chronic insulin treatment, cells were preincubated for 2h with 100 mg/L GSPE, which was maintained during the insulin chronic treatment. After these treatments, acute stimulation (30 min) of GSPE (white columns) or insulin (black columns) was performed and glucose uptake was analyzed. Grey columns indicate basal glucose uptake. Each value represents the mean±S.E.M. of three experiments run in triplicate. Different letters indicate statistically significant differences between treatments ($P<.05$).

parameters). In contrast, Pparg2 remained unaffected. The liver still seemed to be insulin responsive, since the ratio glucose-6-pase vs. glucokinase was lower than that of the controls (Glc6pase: 0.52±0.09; Gck 0.97±0.25, relative expression vs. control). Twenty-five milligrams of GSPE/kg bw per day did not have any effect on liver (G6p: 0.63±0.09; Gck: 0.95±0.14 at 30 days) but was very effective on mesenteric WAT (Fig. 4B). In the latter, the GSPE did not modify any of the first proteins involved in insulin signaling (Irs1) compared to the cafeteria diet. However, we found that GSPE modulated representative markers of mature adipocyte (Glut4, Srebp1c, Pparg2): after 10 days, GSPE seemed to partially revert cafeteria-induced down-regulation of such genes, while a longer treatment (30 days) induced a stronger down-regulation of all these genes. The higher dose, 50 mg of GSPE/kg bw per day (Fig. 4C), showed a similar profile with an even stronger down-regulation of genes after 30 days of treatment. In the liver, this dose was not effective after 10 days of

Table 1
Effects of chronic GSPE treatment on mRNA levels in insulin-resistant 3T3-L1 adipocytes

Chronic treatment	2 h control+15 h insulin 1 nM	2 h GSPE 100 mg/L+15 h insulin 1 nM		
Acute treatment	-	-	Insulin 100 nM	GSPE 100 mg/L
Glut4	1.17±0.16 a	0.54±0.15 b	0.44±0.10 b	0.57±0.02 b
Irs1	1.10±0.18 a	0.30±0.02 b	0.45±0.08 b	0.24±0.02 b
Adiponectin	1.02±0.21 a	0.36±0.03 b	0.31±0.05 b	0.31±0.02 b
Resistin	1.23±0.44 a	0.30±0.10 b	0.24±0.12 b	0.23±0.10 b
Pparg2	0.52±0.13 b	0.02±0.00 c	0.02±0.01 c	0.02±0.00 c
Cebpa	0.36±0.03 b	0.01±0.00 b	0.01±0.00 b	0.01±0.00 b

Fully differentiated adipocytes were given the chronic treatment indicated in the first row. During the last 30 min, each group of adipocytes received the acute treatment indicated at the second row. At the end of treatment, RNA was extracted to quantify gene expression using real time PCR. mRNA gene expression is expressed relative to the control group. Each value represents the mean±S.E.M. Different letters indicate the statistically significant differences between treatments ($P<.05$).

treatment, nor did it favor glucose uptake (Gck: 1.54±0.17; G6p: 0.52±0.02), although it suggested a stimulation of glucose production after 30 days (Gck: 1.67±0.40; G6p: 1.56±0.16).

4. Discussion

Flavonoids and, more specifically, procyanidins are antihyperglycemic in some experimental conditions, thus their potential value as antidiabetics makes them worth studying further. However, there is no consensus on the chronic effects of procyanidins on the maintenance of whole-body glucose homeostasis, in part due to the relatively scarce work done in this area.

Our in vitro results showed different responses to GSPE depending on the assayed model of IR. Under a chronic insulin insult, GSPE showed a slightly improved efficacy at stimulating glucose uptake compared to insulin. This result suggest that GSPE could be useful to stimulate glucose uptake on an animal model of hyperinsulinemia, but also it is a clue to understand mechanistic aspects of this effect, since the different models of IR assayed reflect different alterations of

Table 2
Animals were fed a cafeteria diet for 13 weeks, and respective GSPE doses (25 and 50 mg of GSPE/kg bw per day, labelled +25 and +50, respectively) were administered simultaneously to the cafeteria diet for 10 days (A) and 30 days (B)

A. Effects of 10 days of GSPE treatment on weight and plasma parameters

10 days	Control	Cafeteria	+25	+50
body weight (g)	281.0±9.0 a	393.8±27.0 b	379.7±21.0 b	359.8±17.0 b
visceral adipose tissue (g)	20.0±3.0 a	57.8±7.1 b	54.0±6.5 b	51.6±5.7 b
Leptin (ng/ml)	8.80±1.47 a	35.15±3.18 b	28.89±3.68 b	26.64±4.44 b
HOMA-IR	0.14±0.02 a	6.24±2.18 b	3.24±1.40 a,b	3.87±1.76 a,b
Glucose (mM)	3.32±0.11 a	3.45±0.23 a	4.79±0.13 b	3.66±0.12 b
Insulin (ng/ml)	0.04±0.02 a	1.42±0.44 b	0.61±0.23 a	0.95±0.44 b

B. Effects of 30 days of GSPE-treatment on weight and plasma parameters

30 days	Control	Cafeteria	+25	+50
body weight (g)	274.5±10.0 a	430.2±18.0 b	408.5±9.5 b	426.8±27.0 b
visceral adipose tissue (g)	16.29±0.5 a	67.88±5.3 b,c	58.74±3.8 c	65.17±6.5 b
Leptin (ng/ml)	7.97±0.17 a	38.79±2.63 b	32.60±2.80 b	39.50±2.15 b
HOMA-IR	0.26±0.6 a	9.06±3.8 b	4.16±3.9 a,b	5.35±2.0 b
Glucose (mM)	3.58±0.12 a	3.76±0.10 a	3.81±0.31 a	3.47±0.13 a
Insulin (ng/ml)	0.06±0.15 a	2.13±0.84 b	0.77±0.71 a,b,c	1.28±0.51 c,b

Body weight, WAT weight and leptin levels were obtained on the sacrifice day. Glucose and insulin were measured in fasting condition 3 days before the sacrifice. Each value represents the mean±S.E.M. Different letters indicate statistically significant differences between treatments ($P<.05$).

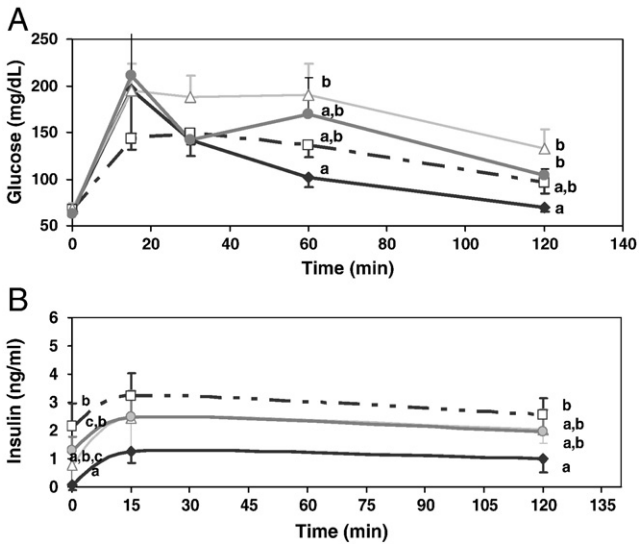


Fig. 3. Plasma glucose and insulin profiles after an intraperitoneal glucose tolerance test (IPGTT) on 30 day GSPE -treated animals. Rats were fasted overnight, a blood sample was collected by tail bleeding (time zero), and glucose was administered intraperitoneally. Blood samples were taken at the time points indicated in the figure. Glucose levels were quantified using a glucometer and insulin using the ELISA method. Data are the mean±S.E.M. of six animals. (A) Glucose profile. (B) Insulin profile. Different letters indicate statistically significant differences between treatments (P<.05).

insulin signaling pathway. Hoehn et al. [28] used the same insults in 3T3-L1 cells and described their main targets. According to their study, chronic insulin was the only insult involving Irs1. The fact that GSPE has a stronger acute effect on this condition suggests that GSPE depends only partially on Irs1 to stimulate glucose uptake. This is in agreement with our previous results showing that the oligomeric components of GSPE activate insulin receptor [29]; however, this is not the only mechanism it has for stimulating glucose uptake [30]. In contrast, we found GSPE to be less effective at acutely stimulating glucose uptake under TNF-α and especially under dexamethasone treatments. TNF-α and dexamethasone induce IR at Glut4 level [28]. Moreover, dexamethasone induces IR through up-regulation of the MAPK phosphatases 1 and 4, resulting in p38 MAPK dephosphorylation, which limited Glut4 activity [31]. We have shown that GSPE depends on p38 to activate glucose uptake [30] and also that the ability of oligomeric procyanidins to activate p38 was higher than its ability to activate de insulin receptor [29]. These evidences suggest GSPE affects the Glut4 transporter itself, which could be by direct interaction or by modulating its activity via p38. In fact, some other flavonoids act directly on glucose transporters, but until present, all the studies reported an inhibitory effect [32,33].

According to cell culture results, we assayed the chronic effects of GSPE in vivo on a disturbed metabolic situation that resembled the most prevalent situation in Western societies and where the main IR factor was hyperinsulinemia. A slight improvement in the plasma IR

Table 3
Acute GSPE effects on metabolite and hormone plasma levels after 30 days of chronic GSPE treatment

Treatment	Control	Cafeteria	+ 25	+ 50
Glucose (mM)	6.93±0.15 a	8.46±0.32 b	7.74±0.22 a,b	8.09±0.37 a,b
Insulin (ng/ml)	1.04±0.21 a	5.42±0.8 b	4.12±0.95 a,b	3.63±0.79 a,b

Animals were fed a cafeteria diet for 13 weeks and were then administered two GSPE doses (25 and 50 mg of GSPE/kg bw per day, labelled +25 and +50, respectively) simultaneously with the cafeteria diet for 30 days. 5h after the last GSPE treatment, the animals were sacrificed and glucose and insulin plasma levels were measured. Each value represents the mean±S.E.M. Different letters indicate statistically significant differences between treatments (P<.05).

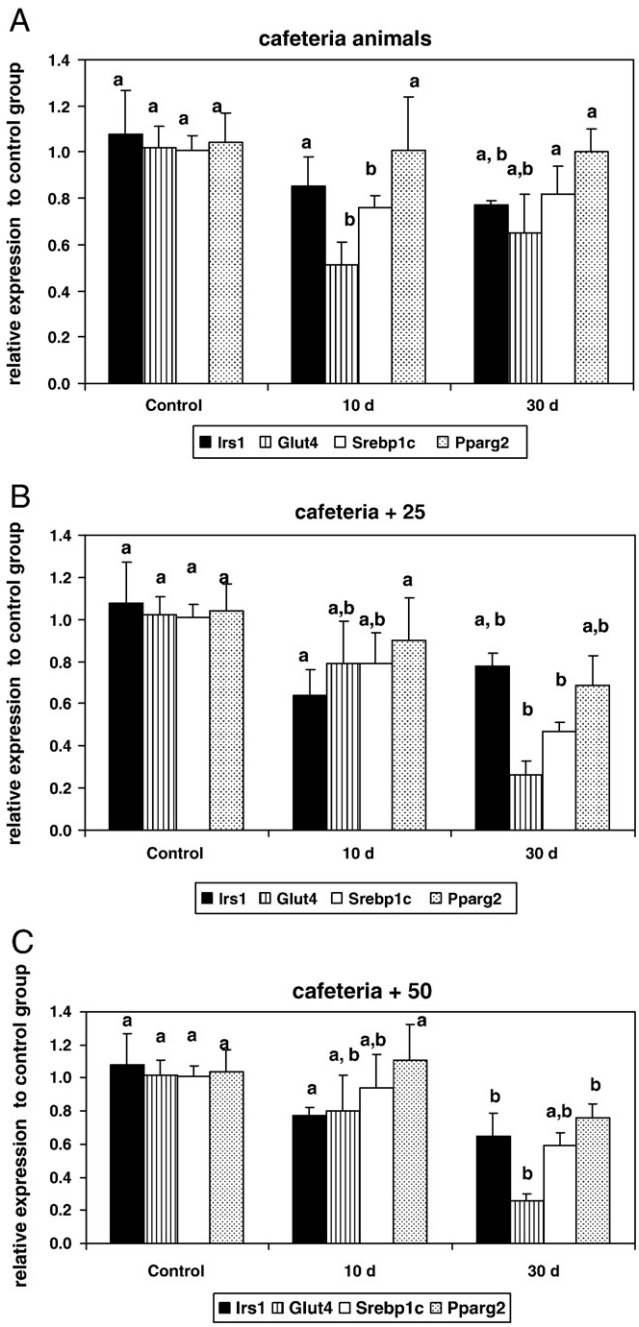


Fig. 4. Gene expression in mesenteric adipose tissue from animals treated with GSPE. After 10 or 30 days of GSPE treatment the animals were sacrificed, and adipose tissue samples obtained. mRNA expression of indicated genes was measured using real time PCR. Data are the mean±S.E.M. of six animals. (A) Effects of the cafeteria diet. (B) Effects of the cafeteria diet + 25 mg GSPE/kg bw per day. (C) Effects of the cafeteria diet + 50 mg GSPE/kg bw per day. Different letters indicate statistically significant differences between treatments (P<.05).

parameters was the main result of these chronic GSPE treatments, after long-term (30 days) treatment. This is the first study to evaluate the effects of GSPE on cafeteria diet-induced IR. Al-Awwadi et al. [17] used a fructose insulin-resistant model to assay the effects of the same dose of grape-derived procyanidin extracts that we used (25 mg/kg bw per day) but for a longer period (6 weeks). They found a slight effect on plasma glucose. Persimmon peel procyanidins at a dose of 10 mg/kg of bw per day for 6 weeks on db/db mice only improved glycosylated protein [18]. All these results suggest that procyanidins

have a moderate effect on improving plasma IR, but only after long-term treatment. In the short treatment, there was some improvement on insulinemia, although long-term treatment was more effective as a whole.

These previous studies showed that procyanidins improved oxidative status and had an anti-inflammatory effect [17,18]. We focused on key points of IR. Our data single out mesenteric white adipose tissue as an important target for GSPE, although GSPE did not clearly improve IR in this tissue. GSPE down-regulated Glut4. GSPE treatment did not improve glucose uptake after the IPGTT. Interestingly, however, the insulin required to maintain glucose levels was lower than in the cafeteria group. This suggests that GSPE could act as insulinomimetic. GSPE would probably act more slowly than insulin because, although we did not find an improvement in glycemia 2 h after the intraperitoneal dose, we did find an improved HOMA-IR in the fasting situation. Thus, we suggest that GSPE acts on adipose cells despite its lower Glut4 mRNA levels. Glut4 has classically been regarded as marker for IR [34]. However, we found in vitro that a long-term GSPE treatment down-regulated Glut4 mRNA but did not modify the glucose uptake response to acute GSPE or insulin treatment. This and previous studies show that adipocytes can acutely respond to glucose uptake signaling regardless of reduced Glut4 mRNA levels [35,36]. They also suggest that GSPE does not activate glucose uptake through its effects at transcriptional level but rather through a different mechanism. As deduced from the in vitro studies, mentioned in the first paragraph of the discussion, this could involve modulating Glut4 self-activation. Furthermore, we did not find significant gene expression modulation either in muscle (results not shown) or liver that could help to explain the effects of GSPE, and this reinforces the specificity of white adipose tissue as target of GSPE. The lack of glucose homeostasis by GSPE in liver differs from other closely related studies. Several flavonoids induce expression of hepatic Gck mRNA or a decrease in mRNA of G6p [7–9,19]. Previously, we also found increased Gck and decreased G6p mRNA levels after acute GSPE treatment of STZ-animals [37]. The different molecules assayed or different metabolic situations might explain the divergences between these studies and our present results. In the present study, we found that GSPE did not affect glucose entrance/output in the liver at the three lower doses, but at the highest dose (50 mg/kg bw per day for 30 days), it did activate G6p, suggesting a negative effect on glucose homeostasis and highlighting the importance of the dose assayed. This is an important point that needs to be solved, but at the present, the lack of studies on this area makes difficult to further analyze the relationship dose-effect about this.

We also found a strong down regulation on markers of mature white adipose tissue, which was not fully matched by a reduction in adipose tissue. We previously found down-regulation of in vitro adipose markers that suggested the adipocytes tended to lose their terminally differentiated state after chronic GSPE treatment [23]. In this study, we show that this also takes place in vivo in an adipogenic milieu (cafeteria diet). Similarly, Cyanidin 3-*o*-*b*-D-glucoside reduces WAT and body weight in mice when administered simultaneously to a 30% high-fat diet at a high dose (200 mg/kg bw) for 12 weeks [6]. Mice fed a high-fat diet (60% energy as fat) and submitted to EGCG treatment (3.2 g/kg diet) also take 16 weeks to reduce the body weight gained and the visceral fat weight [38]. In the present study, GSPE treatment probably needed longer to change the size of adipose depots and thereby influence IR.

In conclusion, in vitro GSPE differed from insulin in how it stimulated glucose uptake in insulin resistant adipocytes. This suggests that GSPE is partly dependent on Irs1 when stimulating glucose uptake but also suggests that GSPE has a direct effect on Glut4 transporter activity. These mechanisms could be responsible for slightly improving plasma IR parameters (mainly by reducing insulinemia). This is achieved by a 25 mg/kg bw per day GSPE dose administered for 30 days on a cafeteria diet-induced impaired glucose tolerance model. This dose did not modify key points of hepatic glucose metabolism (Gck or G6p) but provoked a strong down-regulation on mature adipocyte markers that might be independent of the direct effects of GSPE on Glut4.

Acknowledgments

Thanks are given to members of Nutrigenomics group that have collaborated with caring for and sampling the animals.

References

[1] Kashyap SR, DeFronzo RA. The insulin resistance syndrome: physiological considerations. Diab Vasc Dis Res 2007;4:13–9.
[2] Moller DE, Kaufman KD. Metabolic Syndrome: a clinical and molecular perspective. Annu Rev Med 2005;56:45–62.
[3] Hollander Jason M, Mechanick Jeffrey I. Complementary and alternative medicine and the management of the metabolic syndrome. J Am Diet Assoc 2008;108:495.
[4] Taubert D, Roesen R, Lehmann C, Jung N, Schomig E. Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: a randomized controlled trial. JAMA 2007;298:49–60.
[5] Diepvens K, Kovacs EMR, Vogels N, Westerterp-Plantenga MS. Metabolic effects of green tea and of phases of weight loss. Physiol Behav 2006;87:185–91.
[6] Tsuda T, Horio F, Uchida K, Aoki H, Osawa T. Dietary cyanidin 3-*O*-{beta}-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. J Nutr 2003;133:2125–30.
[7] Jung U, Lee M, Park Y, Kang M, Choi M. Effect of citrus flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. Int J Biochem Cell Biol 2006;38:1134–45.
[8] AePark S, Choi M, Cho S, Seo J, Jung U, Kim M, et al. Genistein and daidzein modulate hepatic glucose and lipid regulating enzyme activities in C57BL/KsJ-db/db mice. Life Sci 2006;79:1207–13.
[9] Wolfram S, Raederstorff D, Preller M, Wang Y, Teixeira S, Riegger C, et al. Epigallocatechin gallate supplementation alleviates diabetes in rodents. J Nutr 2006;136:2512–8.
[10] Potenza MA, Marasciulo FL, Tarquinio M, Tiravanti E, Colantuono G, Federici A, et al. EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR. Am J Physiol Endocrinol Metab 2007;292:E1378–87.
[11] Rivera L, Morón R, Sánchez M, Zarzuelo A, Galisteo M. Quercetin ameliorates metabolic syndrome and improves the inflammatory status in obese Zucker rats. Obesity 2008;16:2081–7.
[12] Pedrielli P, Skibsted LH. Antioxidant synergy and regeneration effect of quercetin, (−)-epicatechin, and (+)-catechin on α-tocopherol in homogeneous solutions of peroxidating methyl linoleate. J Agric Food Chem 2002;50:7138–44.
[13] Maritim A, Dene BA, Sanders RA, Watkins III JB. Effects of pycnogenol treatment on oxidative stress in streptozotocin-induced diabetic rats. J Biochem Mol Toxicol 2003;17:193–9.
[14] Al-Awwadi N, Azay J, Poucheret P, Cassanas G, Krosniak M, Auger CA, et al. Antidiabetic activity of red wine polyphenolic extract, ethanol, or both in streptozotocin-treated rats. J Agric Food Chem 2004;52:1008–16.
[15] Lee Y, Kim Y, Cho E, Yokozawa T. Ameliorative effects of proanthocyanidin on oxidative stress and inflammation in streptozotocin-induced diabetic rats. J Agric Food Chem 2007;55:9395–400.
[16] Pinent M, Blay M, Blade MC, Salvado MJ, Arola L, Ardevol A. Grape seed-derived procyanidins have an antihyperglycemic effect in streptozotocin-induced diabetic rats and insulinomimetic activity in insulin-sensitive cell lines. Endocrinology 2004;145:4985–90.
[17] Al-Awwadi N, Araiz C, Bornet A, Delbosc S, Cristol J-P, Linck N, et al. Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats. J Agric Food Chem 2005;53:151–7.
[18] Lee YA, Cho EJ, Yokozawa T. Effects of proanthocyanidin preparations on hyperlipidemia and other biomarkers in mouse model of Type 2 diabetes. J Agric Food Chem 2008;56:7781–9.
[19] Cao H, Hininger-Favier I, Kelly MA, Benaraba R, Dawson HD, Coves S, et al. Green tea polyphenol extract regulates the expression of genes involved in glucose uptake and insulin signaling in rats fed a high fructose diet. J Agric Food Chem 2007;55:6372–8.
[20] Ardévol A, Bladé C, Salvadó MJ, Arola L. Changes in lipolysis and hormone-sensitive lipase expression caused by procyanidins in 3T3-L1 adipocytes. Int J Obes 2000;24:319–24.
[21] Pinent M, Bladé M, Salvadó M, Blay M, Pujadas G, Fernàndez-Larrea J, et al. Procyanidin effects on adipocyte-related pathologies. Crit Rev Food Sci Nutr 2006;46:543–50.
[22] Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from

fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28: 412–9.
[23] Pinent M, Bladé MC, Salvadó MJ, Arola L, Ardévol A. Intracellular mediators of procyanidin-induced lipolysis in 3T3-L1 adipocytes. J Agric Food Chem 2005;53: 262–6.
[24] Thomson MJ, Williams MG, Frost SC. Development of insulin resistance in 3T3-L1 adipocytes. J Biol Chem 1997;272:7759–64.
[25] Medina EA, Afsari RR, Ravid T, Castillo SS, Erickson KL, Goldkorn T. Tumor necrosis factor-{alpha} decreases akt protein levels in 3T3-L1 adipocytes via the caspase-dependent ubiquitination of Akt. Endocrinology 2005;146: 2726–35.
[26] Sakoda H, Ogihara T, Anai M, Funaki M, Inukai K, Katagiri H, et al. Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. Diabetes 2000;49:1700–8.
[27] Tirosh A, Potashnik R, Bashan N, Rudich A. Oxidative stress disrupts insulin-induced cellular redistribution of insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-L1 adipocytes. A putative cellular mechanism for impaired protein kinase B activation and GLUT4 translocation. J Biol Chem 1999;274:10595–602.
[28] Hoehn KL, Hohnen-Behrens C, Cederberg A, Wu LE, Turner N, Yuasa T, et al. IRS1-independent defects define major nodes of insulin resistance. Cell Metab 2008;7: 421–33.
[29] Montagut G, Onnockx S, Vaqué M, Bladé C, Blay M, Fernández-Larrea J, et al. Oligomers of GSPE activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin. J Nutr Biochem 2009 [In Press, Corrected Proof].
[30] Pinent M, Bladé M, Salvadó M, Arola L, Ardévol A. Metabolic fate of glucose on 3T3-L1 adipocytes treated with grape seed-derived procyanidin extract (GSPE). Comparison with the effects of insulin. J Agric Food Chem 2005;53:5932–5.
[31] Bazuine M, Carlotti F, Rabelink MJWE, Vellinga J, Hoeben RC, Maassen JA. The p38 mitogen-activated protein kinase inhibitor SB203580 reduces glucose turnover by the glucose transporter-4 of 3T3-L1 adipocytes in the insulin-stimulated state. Endocrinology 2005;146:1818–24.
[32] Bazuine M, van den Broek PJ, Maassen JA. Genistein directly inhibits GLUT4-mediated glucose uptake in 3T3-L1 adipocytes. Biochem Biophys Res Commun 2005;326:511–4.
[33] Chen C, Hsu H, Huang Y, Linck N. Interaction of flavonoids and intestinal facilitated glucose transporters. Planta Med 2007;73:348–54.
[34] Abel ED, Peroni O, Kim JK, Kim Y, Boss O, Hadro E, et al. Adipose-selective targeting of the GLUT4 gene impairs insulin action in muscle and liver. Nature 2001;409: 729–33.
[35] Li J, Houseknecht KL, Stenbit AE, Katz EB, Charron MJ. Reduced glucose uptake precedes insulin signaling defects in adipocytes from heterozygous GLUT4 knockout mice. FASEB J 2000;14:1117–25.
[36] Im SS, Kwon SK, Kim TH, Kim HI, Ahn YH. Regulation of glucose transporter type 4 isoform gene expression in muscle and adipocytes. IUBMB Life 2007;59:134–45.
[37] Fernandez-Larrea J, Montagut G, Bladé MC, Salvadó MJ, Blay MT, Pujadas G, et al. GSPE has the same effects as insulin on the mRNA levels of the main genes of glucose disposal in the liver of STZ-diabetic animals. Diab Vasc Dis Res 2007;1: S186.
[38] Bose M, Lambert JD, Ju J, Reuhl KR, Shapses SA, Yang CS. The major green tea polyphenol, (−)-epigallocatechin-3-gallate, inhibits obesity, metabolic syndrome, and fatty liver disease in high-fat-fed mice. J Nutr 2008;138:1677–83.