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Effects of different doses of resveratrol on body fat and serum parameters in rats fed a hypercaloric diet

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Recently resveratrol, a compound naturally occurring in various plants, has been proposed as a potential anti-obesity compound. The aim of the present work was to analyse the effects of different doses of resveratrol on body fat and serum parameters in rats. Thirty-two male Sprague-Dawley rats were randomly divided into four groups and fed on a hypercaloric diet for 6 weeks. The doses of *trans*-resveratrol used were 6, 30 and 60 mg/kg body weight/d in RSV1, RSV2 and RSV3 groups respectively. The stability of resveratrol when added to the diet was evaluated. Blood samples were collected, and white adipose tissue from different anatomical locations, interscapular brown adipose tissue, gastrocnemious muscles and liver were weighed. Commercial kits were used to measure serum cholesterol, glucose, triacylglycerols and non-esterified fatty acids. While the lowest dose did not have a body fat reducing effect, the intermediate dose reduced all the white adipose depots. The highest dose significantly reduced mesenteric and subcutaneous depots but not epididymal and perirenal tissues. Although the reduction in all the anatomical locations analysed was 19% in the RSV3 group, in the RSV2 group it was 24%. No significant differences among the experimental groups were found in brown adipose tissue, gastrocnemious muscle or liver weights. Serum parameters were not affected by resveratrol intake because no differences among the experimental groups were observed. These results suggest that resveratrol is a molecule with potential anti-obesity effect. The most effective of the three experimental doses was 30 mg/kg body weight/d.

Key words: Resveratrol, Body fat, Hypercaloric diet, Rat.

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Obesity is a growing problem worldwide that is reaching epidemic proportions and is associated with an increased mortality risk (1, 11, 27). Although weight loss and weight control drugs are becoming common in modern society, the industry-provided remedies very often have failed in long-term maintenance of weight loss in obese patients. For instance, although in recent years a great deal of work has been performed in order to analyse the body fat-lowering effect of conjugated linoleic acid (CLA), its effectiveness in humans remains controversial and potential deleterious effects are still a matter of concern (15, 18, 25).

Taking this situation into consideration, substantial progress is being made in widening knowledge about biological components in plant foods and their links with obesity (22). In this context polyphenols make up one of the molecule groups most frequently studied in recent years.

Resveratrol (*trans*-3,5,4'-trihydroxystilbene) is a phytolaexin polyphenolic compound occurring naturally in various plants, including grapes, berries and peanuts, in response to stress, as a defence mechanism against fungal, viral, bacterial infections and damage from exposure to ultraviolet radiation (14, 26). Resveratrol exists in *trans* and *cis* stereoisomeric forms, although the *trans* form is more photo- and thermostable than the *cis* form. Standard *trans*-resveratrol solutions also show photoisomerisation: when exposed to daylight, they undergo changes in the *trans* stereoisomer ratio as the amount of the *cis* isomer increases to the detriment of the *trans* forms amount (12, 17).

A remarkable range of biological functions is ascribed to this molecule. For example, it acts as a cancer chemoprevention agent, a powerful anti-inflammatory factor and an antioxidant agent (8, 10). Its cardiovascular properties, including inhibition of platelet aggregation and promotion of vasodilation, by enhancing the production of nitric oxide, have also been described (6). Thus, it seems that resveratrol is involved in the so-called "French paradox", a condition in which the consumption of moderate amounts of red wine (rich in resveratrol) protects against cardiovascular damage (21).

More recently, resveratrol has been proposed as a potential anti-obesity compound. *In vitro* studies performed in 3T3-L1 adipocytes have demonstrated that resveratrol induces apoptosis (23) and increases lipolysis induced by epinephrine (20). Moreover, this molecule inhibits proliferation and differentiation of preadipocytes in 3T3-L1 cells (20) and pig adipocyte cultures (3).

With regard to *in vivo* studies, BAUR *et al.* (4) showed that resveratrol shifted the physiology of middle-aged mice fed on a high-calorie diet towards that of mice fed on a standard diet, and significantly increased their survival. Moreover, LAGOUGE *et al.* (13) found that treatment of mice with resveratrol significantly increased oxygen consumption in muscle fibres due to the induction of genes for oxidative phosphorylation and mitochondrial biogenesis.

The aim of the present work was to analyse the effects of resveratrol on body fat and serum parameters in rats fed on a hypercaloric diet. At present little is known about which doses are effective in achieving some of the beneficial effects reported for this molecule. This being so, the present study tested three doses in order to determine the most effective one.

Material and Methods

Animals, diets and experimental design.– Thirty two male Sprague-Dawley rats (180 ± 2 g) were purchased from Har-

lan Ibérica (Barcelona, Spain). They were individually housed in polycarbonate metabolic cages (Techniplast Gazzada, Guguggiate, Italy) and placed in an air-conditioned room (22 ± 2 ºC) with a 12-h day-night rhythm (light on at 21.00). After a 6-day adaptation period, the animals were fed with a commercial hypercaloric diet (4.6 kcal/g) supplied by Harlan Ibérica (ref. TD.06415) for 6 weeks. The diet consisted of 245 g/kg casein, 3.5 g/kg L-cystine, 85 g/kg corn starch, 115 g/kg maltodextrin, 200 g/kg sucrose, 195 g/kg lard, 30 g/kg soybean oil, 58 g/kg cellulose, 43 g/kg mineral mix, 3.4 g/kg calcium phosphate dibasic, 19 g/kg vitamin mix and 3 g/kg choline bitartrate. All animals had free access to food and water. Food intake and body weight were measured daily and on the last day naso-anal length was measured as an indicator of linear growth.

Resveratrol determination and dosage in the diet.– The stability of *trans*-resveratrol was first checked when homogenised into the diet and kept at 22 ± 2 ºC, protected from light in the same conditions used in the metabolic cage feeders. Its concentration was determined by HPLC-DAD (Agilent 1200 system). A reversed-phase Gemini (Phemomenex) C18 (250 x 4.6 mm I.D., 5µm) was used. Solvents for the mobile phase were water (A) and methanol (B) both in 0.1% formic acid. The elution conditions applied were: 0-9 min, 50% B isocratic; 9-11 min, linear gradient 50-100% B; 11-13 min, 100% B isocratic; and finally 13-15 min, linear gradient 100-50% for reconditioning the column. The flow rate was 1 mL/min and the injection volume was 50 µL. The chromatographic separation was carried out at 30 ºC. *Trans*-resveratrol was monitored at 305 nm and *cis*-resveratrol at 285 nm. Identification of resveratrol isomers was made possible by comparison of detection times and UV-vis spectra with those of the standards. Quantification was performed by reporting the measured integration areas in the calibration equation of the corresponding standards.

We found that resveratrol degraded almost completely over the feeding time. Therefore we concluded that mixing it in diet, the most common method used to test the biological effects of a functional molecule intake, was not a suitable system.

In previous studies we observed that the rats started eating immediately upon daily diet replacement at the start of the dark period. Thus, we decided to check the stability of resveratrol when added to the surface of the diet. With this aim, an ethanolic solution of resveratrol (5 mg/mL) was added to the diet (0.3 mg resveratrol/g diet) and kept in the same conditions. Resveratrol was extracted from the diet at different times between 0-16 h using a mixture methanol:water: formic acid (70:29,8:0,2) assisted by ultrasonic bath, then measured. We observed that resveratrol concentration in the diet decreased up to 50% in the first 3 hours. However, no further reductions were found after this time (Fig. 1).

Taking into account these results, and considering that the rats started eating immediately when the diet was replaced, this system was used in order to ensure the amount of resveratrol that the animals consumed.

For this study rats were randomly divided into four groups: C, RSV1, RSV2, RSV3. Due to its low solubility in water, resveratrol was dissolved in ethanol (30 mg/mL). The amount of resveratrol solution added to the diet was daily adjusted according to the animal weight to ensure the following doses: 6 mg resveratrol/kg body weight/d in RSV1 group, 30 mg resveratrol/kg kg body weight/d in RSV2 group, and 60 mg resveratrol/kg body

weight/d in RSV3 group. In order to avoid differences in the amount of ethanol received by each animal, ethanol was added to the diet to reach 2 mL/kg body weight/d. Rats in C group received this amount of ethanol without resveratrol daily.

Tissue removal and serum sampling.- At the end of the experimental period blood samples were collected under anaesthesia (chloral hydrate) by cardiac puncture. White adipose tissue (WAT) from different anatomical locations (epididymal, perirenal, mesenteric and subcutaneous), brown adipose tissue from interscapular area (IBAT), gastrocnemious muscles and liver were dissected, weighed and immediately frozen. Serum was obtained from blood samples after centrifugation (1000*g* for 10 min at 4 °C). All samples were stored at -80 °C until analysis.

Commercial kits were used to measure serum cholesterol (BioSystems; Barcelona, Spain), glucose (BioSystems; Barcelona, Spain), triacylglycerols (Spinreact; Sant Esteve de Bas, Spain) and non-esterified fatty acids (NEFA) (Roche; Penzberg, Germany).

Statistical analysis.- Results are presented as mean ± S.E.M. Statistical analysis was performed using SPSS 16.0 (SPSS, Chicago, IL, USA). Data were analysed by one-way ANOVA followed by Newman Keuls *post hoc* test. Significance was assessed at the $P<0.05$ level.

Results

Food intake, body weight and length, and tissue weights.– No statistical differences in food intake or in final body weight were found among the experimental groups. Body length, used as an indicator of body growth was not modified (Table I).

Resveratrol reduced the size of the white adipose tissues analysed in four different locations. Although a clear dose response was not observed, important differences were found among the three experimental doses. Thus, the lowest dose (6 mg/kg/d) did not show a body fat reducing effect, whereas the intermediate dose (30 mg/kg/d) reduced all the adipose depots. Surprisingly, the highest dose (60 mg/kg/d) led to significantly reduced mesenteric and subcutaneous depots but

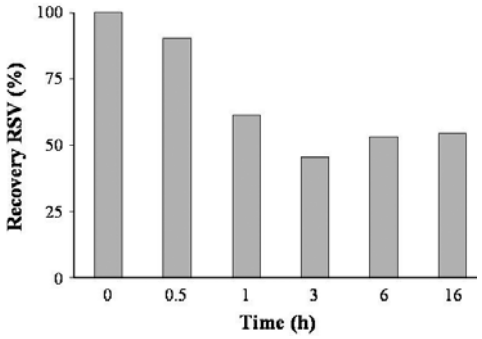

Fig. 1. *Resveratrol (RSV) degradation over time when added to the surface of the diet in an ethanolic solution.*

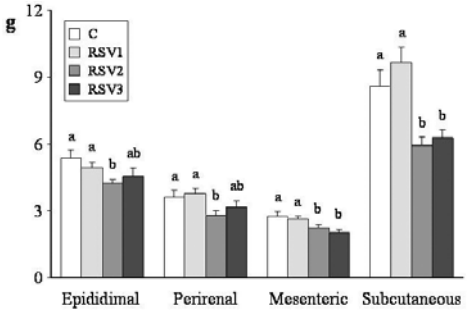

Fig. 2. *White adipose tissue weights in control and resveratrol-treated rats.*
Values are means ± SEM (n = 8). Values not sharing a common letter are significantly different ($P < 0.05$).

Table I. *Food intake, final body weight, body length, tissue weights (interscapular brown adipose tissue, gastrocnemious muscles and liver), and serum parameters of control and resveratrol-treated rats*

	C	RSV1	RSV2	RSV3
Food intake (g/d)	13.8 ± 0.4	14.4 ± 0.3	13.7 ± 0.3	14.3 ± 0.3
Final body weight (g)	341 ± 11	344 ± 7	332 ± 7	345 ± 9
Body length (cm)	22.6 ± 0.3	23.0 ± 0.3	22.4 ± 0.5	22.6 ± 0.3
IBAT (g)	0.71 ± 0.05	0.70 ± 0.06	0.63 ± 0.03	0.68 ± 0.06
Gastrocnemious muscles (g)	2.95 ± 0.28	2.70 ± 0.09	2.79 ± 0.11	2.65 ± 0.06
Liver (g)	11.79 ± 0.64	11.15 ± 0.22	11.64 ± 0.71	12.11 ± 0.53
Serum parameters				
Cholesterol (mmol/L)	2.06 ± 0.11	1.80 ± 0.15	2.08 ± 0.07	2.37 ± 0.16
Triacylglycerols (mmol/L)	1.41 ± 0.22	1.28 ± 0.14	1.67 ± 0.28	1.15 ± 0.12
Free fatty acids (mmol/L)	0.42 ± 0.08	0.30 ± 0.04	0.31 ± 0.05	0.24 ± 0.02
Glucose (mmol/L)	6.00 ± 0.28	6.35 ± 0.33	6.56 ± 0.54	6.63 ± 0.60

Values are means ± SEM (n = 8). IBAT, Interscapular brown adipose tissue. No significant differences among the experimental groups were found in the analyzed parameters.

not epididymal and perirenal tissues (Fig. 2). The reduction in all the anatomical locations analysed was 19% in the RSV3 group, whereas in the RSV2 group it was 24%.

No significant differences were found in interscapular brown adipose tissue, gastrocnemious muscle or liver weights among experimental groups.

Serum parameters.- Serum parameters, cholesterol, triacylglycerols, free fatty acids and glucose, were not affected by resveratrol intake because no differences were observed among experimental groups (Table I).

Discussion

In the present study, the effects of different doses of resveratrol under *in vivo* conditions were analysed. As far as we know, there are no other studies in the literature devoted to comparing the effectiveness of different doses of this molecule on body fat.

Since potential alterations in resveratrol can be induced when this compound is added to the diet, its stability was first of all analysed in order to find the system which best ensured accuracy in the doses provided to the rats. The results revealed that no *cis*-resveratrol was found in the supplemented diet. This suggests that the degradation of *trans*-resveratrol was not related to photoisomerisation reactions. It is well known that resveratrol has a strong antioxidant potential, as it is an excellent scavenger of hydroxyl, superoxide, and other radicals (5,16). The decrease in the concentration of *trans*-resveratrol observed might be due to oxido-reduction reactions between resveratrol and the high-fat enriched diet. Thus, we concluded that mixing the resveratrol in diet, the method most commonly used to test the biological effects of a functional molecule intake, was not a suitable system. Instead, we placed an ethanolic solution of resveratrol on the food surface.

The results here reported show that resveratrol induced a reduction in adipose depot size in rats fed a fattening diet (high-fat, high-sucrose), not due to decreased food intake, as the amount of diet consumed per rat throughout the

experimental period was unchanged. The effects of resveratrol on body fat by using animal models have been little studied to date. Only three studies assessing the effect of resveratrol on body weight and/or body fat have been published. LAGOUGE *et al.* (13) reported that C57BL/6J mice fed on a high-fat diet and treated with 400 mg resveratrol/kg body weight/d showed significantly reduced final body weight after 9 weeks of treatment. This decrease in body mass was accounted for by a decrease in fat and was reflected in the mass of the different white fat pads. AHN *et al.* (2) observed that body weight gain was lower in C57BL/6J mice fed an atherogenic diet supplemented with 0.0125% resveratrol than in mice fed the atherogenic diet without supplementation. In this study, body fat was not assessed. In another experiment conducted in obese Zucker rats a dose of 10 mg resveratrol administered orally did not lead to reduced body weight but reduced 10% body fat content. As in the present study, resveratrol did not modify food intake (24). In general terms, our results are in line with those published by other authors.

Interestingly a dose-response was not found. Thus, while the lowest dose (6 mg/kg body weight/d; RSV1 group) was totally ineffective, the intermediate dose (30 mg/kg body weight/d; RSV2 group) significantly reduced all the adipose tissue analysed. In RSV3 group (60 mg/kg body weight/d) only mesenteric and subcutaneous adipose tissues were significantly reduced. Moreover, when significant reductions were observed, the magnitude of the decrease was no greater than that observed with the intermediate dose. Consequently it can be stated that the most effective of the three experimental doses was 30 mg/kg body weight/d.

The dose of resveratrol that was effective in the present study (30 mg/kg body weight/d) is far higher than the amount usually ingested by humans (100-930 µg/d) (9,28), meaning that the positive effects of this molecule on body fat should be achieved by the intake of resveratrol pills or functional foods enriched with this molecule. Nevertheless, it is important to point out that doses close to that used in the present study are being tested on humans (www.clinicaltrial.gov).

Resveratrol is one of the natural molecules described as a potential treatment agent for diabetes and hyperlipidemia (29). Thus, in the study published by RIVERA *et al*. (24), Zucker rats treated with resveratrol showed reduced plasma triacylglycerols, free fatty acids and glucose, and ameliorated insulin function. These results were not found in the present study because no significant differences in plasma parameters were observed among the four experimental groups.

RIVERA *et al*. (24) explained that the improvement in insulin action, and consequently the reduction in serum glucose levels, as well as the decrease in triacylglycerols, were probably associated with the reduction in free fatty acid levels, an effect that in its turn was due to the reduction adipose tissue. In the present study, although serum free fatty acids tendd to decrease in resveratrol-treated rats, this reduction did not reach any statistical significance. It could be hypothesized that the lack of change in this parameter is based on the lack of change in other serum parameters, such as glucose and triacylglycerols. The reasons for the difference between our study and that of RIVERA *et al*. are not clear. Nevertheless, it should be emphasized that obese Zucker rats have a very important hypertrophy in adipose tissue and that their adipocytes release a

high amount of free fatty acids in the basal state (7, 19). Consequently the reduction in adipose tissue has a great impact on the level of serum free fatty acids. Very probably, in the present study the hypertrophy induced by the hypercaloric diet was lower than that showed by obese Zucker rats and thus the reduction in adipose tissue did not lead to significantly reduced free fatty acid levels.

In conclusion this study suggests that resveratrol is a molecule with a potential anti-obesity effect. The most effective of the three experimental doses analysed was 30 mg/kg body weight/d.

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