Effects of hydroxytyrosol on cardiovascular biomarkers in experimental diabetes mellitus

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Abstract

The aim of this study was to assess the influence of hydroxytyrosol (HT) on cardiovascular biomarkers and morphometric parameters of the arterial wall in

streptozotocin-diabetic rats. Seven groups of rats (N=10 per group) were studied for 2 months: nondiabetic rats (NDR), diabetic rats treated with saline (DR) and DR treated with HT (0.5, 1, 2.5, 5 and 10 mg kg−1 day−1 p.o.). DR had higher platelet aggregation values, higher thromboxane B2, plasma lipid peroxidation, 3-nitrotyrosine, oxidized LDL (oxLDL), myeloperoxidase, vascular cell adhesion molecule 1 (VCAM-1) and interleukin-1β (IL-1β) concentrations, and lower aortic 6-keto-prostaglandin F1α and nitric oxide production than NDR. Aortic wall area and smooth muscle cell count were also higher in DR than in NDR. HT significantly reduced both oxidative and nitrosative stress, oxLDL concentration, VCAM-1 and inflammatory mediators, platelet aggregation and thromboxane B2 production. Morphometric values in the aortic wall were reduced to values near those in NDR. In conclusion, HT influenced the major biochemical processes leading to diabetic vasculopathy, and reduced cell proliferation in the vascular wall in this experimental model.

Abbreviations

NDR, nodiabetic rats; DR, diabetic rats; oxLDL, oxidized low-density lipoprotein; MPOx, myeloperoxidase; IL-1β, interleukin-1β; ROS, reactive oxygen species; HT, hydroxytyrosol; HPLC, high-performance liquid chromatography; 6-keto-PGF1α, 6-keto-prostaglandin F1α; DTT, DL-dithiothreitol; TBARS, thiobarbituric acid reactive substances; GSH, reduced glutathione; SEM, standard error of the mean; Imax, maximum intensity of platelet aggregation; TxB2, platelet thromboxane B2; GSSG, oxidized glutathione

Keywords

1. Introduction

Diabetes mellitus is one of the most prevalent diseases in the world, with an estimated worldwide prevalence of approximately 8% [1]. The basis of treatment for this disease is maintaining blood glucose levels within the limits found in the nondiabetic population. However, complications of diabetes are common and arise from either poor metabolic control (hyperglycemia and hypoglycemia) or prolonged high levels of blood glucose, leading to the development of diabetic vascular complications such as macroangiopathy and microangiopathy.

The mechanisms of diabetic vasculopathy are related to chronic excess blood glucose, which causes intracellular metabolic changes involving the polyol pathway, hexosamine biosynthetic pathway, diacylglycerol-protein kinase C, glycation end products, and the intracellular generation of reactive oxygen species (ROS), among other mechanisms. [2]. Oxidative stress activates other cytotoxic pathways, and ROS formation is considered the earliest biochemical step in the process of endothelial cell damage in

diabetes mellitus. Thereafter, so-called endothelial dysfunction can appear and may in turn give rise to diabetic vasculopathy [3].

Because of the importance of oxidative stress in the onset of endothelial dysfunction in diabetes, a possible preventive approach to diabetic vasculopathy, in addition to controlling blood glucose levels, may be the use of antioxidant compounds [4]. In this connection the Mediterranean diet (rich in antioxidants) has been associated with better metabolic control of diabetes, as well as a lower overall incidence of cardiovascular events [5] ; [6]. Virgin olive oil plays an important role in the beneficial effects of the Mediterranean diet. Hydroxytyrosol (HT), the main polyphenol present in its free form in virgin olive oil, exerts beneficial cardiovascular effects and has been associated with decreased oxidized low-density lipoprotein (oxLDL) and platelet aggregation, and lower production of inflammatory mediators, along with other effects [7] ; [8].

The main objective of this study was to determine the possible influence of HT (0.5–10 mg kg−1 day−1 orally) on cardiovascular biomarkers and morphometric parameters in the arterial wall, in an experimental model of diabetes mellitus. The biomarkers studied here are related to oxidative and nitrosative stress, platelet function, prostanoid synthesis, adhesion molecules and inflammatory mediators involved in diabetic vasculopathy.

2. Material and methods

2.1. Material

Thromboxane B2, interleukin-1β (IL-1β), 3-nitrotyrosine and 6-keto-prostaglandin F1α (6-keto-PGF1α) enzyme immunoassay kits were from GE Healthcare UK (Little Chalfont, Buckinghamshire, UK). The nitrite/nitrate ELISA kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). Collagen was obtained from Menarini Diagnóstica (Barcelona, Spain). All other reagents were from Sigma Chemical Corp. (St. Louis, MO, USA).

Hydroxytyrosol was isolated by hydrothermal treatment of the liquid phase obtained from alperujo (a by-product of the two-phase olive oil separation system) at 160 °C for 60 min [9]. The liquid was extracted by chromatography fractionation in two steps, with a final yield of 99.6% purity referred to dry matter, according to the process described by Fernández-Bolaños et al. [10]. For high-performance liquid chromatography (HPLC), standard tyrosol compound was obtained from Fluka (Buchs, Switzerland) and HT from Extrasynthese (Lyon Nord, Geney, France). The phenols were quantified using a Hewlett-Packard 1100 liquid chromatography system with an ultraviolet–visible detector. A Mediterranean sea C18 analytical column (250×4.6 mm i.d.; particle size=5 μm) (Teknokroma, Barcelona, Spain) was used at room temperature. The system was equipped with Rheodyne injection valves (20 μl loop). The mobile phases were 0.01% trichloroacetic acid in water and acetonitrile, with the following gradient during a total run time of 55 min: 95% A initially, 75% at 30 min, 50% at 45 min, 0% at 47 min, 75% at 50 min, and 95% at 52 min until the run was complete. Quantification was carried out by peak integration at 280 nm wavelength with reference to calibrations obtained with external standards. Fig. 1 shows a representative chromatographic profile of HT purified after thermal treatment.

2.2. Study design

The animals were 2-month-old adult male Wistar rats (body weight 200–250 g). All rats were used in accordance with current Spanish legislation for animal care, use and housing (EDL 2013/80,847. BOE-A-2013-6271). The recommendations in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86–23, revised 1985) were followed, as well the Spanish Law on the Protection of Animals, where applicable. The study protocol was approved by the University of Malaga Ethics Committee for the Use of Animals.

Animals (10 rats per group) were distributed in seven groups: 1) control nondiabetic rats (NDR) treated with saline, 2) control diabetic rats (DR) treated with saline, 3) DR treated with 0.5 mg kg−1 day−1 p.o. HT, 4) DR treated with 1 mg kg−1 day−1 p.o. HT, 5) DR treated with 2.5 mg kg−1 day−1 p.o. HT, 6) DR treated with 5 mg kg−1 day−1 p.o. HT, and 7) DR treated with 10 mg kg−1 day−1 p.o. HT. These doses were chosen according to results obtained previously with HT to analyze some of the biomarkers quantified in this study in healthy animals. Hydroxytyrosol was given once per day for 7 days before diabetes was induced, and then daily until the end of the diabetic period (2 months) via an endogastric cannula (daily gastric intubation to asses HT amount in each dose) at 10:00 am.

Experimental diabetes was induced with a single intravenous injection of streptozotocin (50 mg/kg). Blood glucose concentration was measured by placing a Glucocard

Memory II glucosimeter (Menarini, Barcelona, Spain) in contact with blood from the saphenous vein. Animals were considered to have diabetes if blood glucose was higher than 200 mg/dl for 2 consecutive days. Rats in the nondiabetic control group received a single intravenous injection of isotonic saline solution, and blood glucose was measured in the same way as in diabetic animals. Even knowing that no method of induction of experimental diabetes is totally comparable to human diabetes, we have chosen induction with streptozotocin in order to assess the best possible type 1 diabetes and for being the method least amount of ROS produced [11], which it is important because we use in this study a demonstrated a compound with antioxidant properties.

During the treatment up period, diabetic animals were treated with 4 IU/day s.c. of a soluble long-acting basal insulin analog (Levemir®, Novo Nordisk España, Madrid, Spain) to reduce mortality due to the high levels of blood glucose. Control animals received the same volume of isotonic saline solution s.c.

At the end of the second month, all animals from each group were anesthetized with pentobarbital sodium (40 mg/kg i.p.). A medial laparotomy was made to withdraw 2 ml of blood from the vena cava; 3% sodium citrate at a proportion of 1:9 was used as the anticoagulant. Then a segment of the abdominal aorta 0.5 cm anterior to the bifurcation of the femoral arteries was obtained.

2.3. Analytical techniques

All techniques were run in a single-blind manner, i.e., the persons who did the assays were unaware of the origin and nature of the samples.

2.3.1. Platelet aggregometry

Platelet aggregation capacity in whole blood was tested at 37 °C with the electrical impedance method (Chrono-Log 540 aggregometer, Chrono-Log Corp., Haverton, PA, USA). Collagen (10 μg/ml) was used as the aggregation inducing agent. Maximum aggregation intensity was determined as the maximum resistance between the two poles of the electrode obtained 10 min after the agonist was added.

2.3.2. Platelet thromboxane B2

After aggregation was complete the blood sample was centrifuged at 10,000×g for 5 min, and the supernatant was frozen at−80 °C until thromboxane B2 production was quantified with an enzyme immunoassay.

2.3.3. Vascular 6-keto-prostaglandin F1α

The aortic segment was cut into two parts and incubated at 37 °C in buffer containing (mM): 100 NaCl, 4 KCl, 25 NaHCO3, 2.1 Na2SO4, 20 sodium citrate, 2.7 glucose and 50 Tris (pH 8.3). Segments were placed in 500 μl fresh buffer, and 10 μl calcium ionophore A23187 (final concentration 50 μM) was added. Thirty minutes later the samples were dried and weighed, and the supernatant was frozen at−80 °C until the

assay. The production 6-keto-PGF1α (stable metabolite of prostacyclin) was quantified with an enzyme immunoassay.

2.3.4. Vascular nitric oxide production

Nitric oxide production was induced with the calcium ionophore A23187 (50 μM) and measured as nitrite + nitrate concentration in the supernatant of induced aortic rings with an enzyme immunoassay kit. Briefly, aortic rings were incubated for 10 min at 37 °C with 200 μl buffer containing 10 mM HEPES, 1 mM EDTA, 1 mM DL-dithiothreitol (DTT), 10 μg ml-1 leupeptin, 4.8 mM DL-valine, 1 mM NADPH, 1 mM MgCl2, 1 mM CaCl2 and 20 μM L-arginine. Basal nitrite + nitrate production was first determined; after this step calcium A 23187 ionophore was added and after 30 min of incubation at 37 °C the increase in nitrite + nitrate production was measured.

2.3.5. Plasma lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were measured as an index of plasma lipid peroxide concentration. Samples of plasma were incubated with 500 μl 0.5% thiobarbituric acid in 20% trichloroacetic acid. The samples were shaken and incubated at 100 °C for 15 min, then centrifuged at 2000×g for 15 min at 4 °C. Absorbance of the resulting supernatant was determined spectrophotometrically at 532 nm (FluoStar, BMG Labtechnologies, Offenburg, Germany). Blank samples were prepared in an identical manner except that they were incubated at 4 °C in order to avoid TBARS production.

2.3.6. Erythrocyte glutathione concentration

Reduced glutathione (GSH) was measured in red blood cells after they were homogenized in 0.1 M sodium phosphate buffer (pH 8.0) with 25% phosphoric acid, then centrifuged at 13,000×g for 15 min at 4 °C to obtain the supernatant. Duplicate cuvettes were prepared for spectrofluorometry with sodium phosphate buffer, the supernatant for each sample, and o-phthaldehyde. Measurements were made at 350 nm excitation wavelength and 440 nm emission wavelength. To determine the proportions of oxidized and reduced glutathione we incubated the supernatant from each sample with 4-vinylpyridine, then proceeded as for as total glutathione.

2.3.7. Plasma 3-nitrotyrosine and oxidized low-density lipoprotein cholesterol

Plasma levels of 3-nitrotyrosine were measured as an indirect index of peroxynitrite production. oxLDL was measured as one of the most important biomarkers in the first stages of cardiovascular disease. Plasma was obtained and frozen at−80 °C until the assay. The production of 3-nitrotyrosine and oxLDL was quantified with an enzyme immunoassay.

2.3.8. Serum interleukin 1β, myeloperoxidase and VCAM-1

Two milliliters of native blood was incubated at 37 °C for 30 min, then serum was obtained after centrifugation at 2500×g for 15 min at 4 °C. Serum was frozen at−80 °C until the assay. The production of interleukin 1β, myeloperoxidase (MPOx) and VCAM-1 was quantified with an appropriate enzyme immunoassay.

2.3.9. Aortic morphometric analysis

Morphological analyses were done in a segment of aorta from the division of the renal arteries to 1 cm upwards. The excised tissue was fixed in 10% paraformaldehyde by total immersion for 48 h and was processed with the classical paraffin embedding method. Paraffin-embedded sections were cut at 7 μm and stained with hematoxylin and eosin. The sections were examined under a research microscope equipped with a digital system. Histomorphometric analysis was carried out with the Visilog v. 6.3 computer program licensed to the Central Computer Service of the University of Malaga.

From each arterial sample, 10 randomly chosen sections from 5 to 7 slides (containing 5–8 sections per slide) were analyzed. In each section we quantified the variables (i) area of the lumen (AL) and (ii) area of the entire arterial section (AW). The area of the arterial wall (AAW) was calculated as follows:

AAW=AW-AL

In addition, stained aortic sections were used to count the number of smooth muscle cell nuclei in the tunica media. The image was segmented into a new binary image, with black representing the nuclei. Before calculation, any artifacts or particles smaller than the predetermined size were eliminated. The number of cell nuclei was then calculated within four fields of approximately 10,000 μm2 at 0°, 90°, 180° and 270° in each section.

2.4. Statistical analysis

The data in the text, tables and figures are expressed as the mean ± standard error of the mean of 10 animals. All statistical analyses were done with the Statistical Package for Social Sciences v. 23.0 (SPSS Co., Chicago, IL, USA). One-way analysis of variance followed by Bonferroni transformation and unpaired Student's t tests were used. To establish possible relationships between morphological data from aorta sections and biochemical variables, Pearson correlation coefficients were calculated. In all cases, statistical significance was assumed at a value of $P<.05$.

3. Results

Mean body weight, blood glucose concentration and blood cell counts after 2 months are shown in Table 1. There were no statistically significant differences in body weight or blood cell counts between groups.

Regarding platelet variables, diabetic rats had a 95% higher platelet reactivity to collagen and a 69% higher serum thromboxane B2 concentration than nondiabetic rats (Table 2). The chronic administration of HT reduced platelet reactivity in diabetic rats in a dose-dependent manner (from a 7% reduction with 0.5 mg kg−1 day−1 to a 62% reduction with 10 mg kg−1 day−1). Serum thromboxane B2 concentration was also reduced in a dose-dependent manner after the administration of HT (from an 11% reduction with 0.5 mg kg−1 day−1 to a 38% reduction with 10 mg kg−1 day−1).

Regarding biochemical vascular variables (Table 3), calcium-induced 6-keto-PGF1α production (an indirect index of prostacyclin production) was 55% lower in diabetic animals than in normoglycemic rats; chronic HT administration increased these values to near those in nondiabetic animals at doses from 2.5 to 10 mg kg−1 day−1. Calcium-induced nitric oxide production, measured as nitrite plus nitrate formation (Table 3), was 42% lower in diabetic animals than in normoglycemic rats; HT significantly increased this variable only at a dose of 10 mg kg−1 day−1.

Diabetic rats showed a more pronounced imbalance in blood oxidative stress variables than nondiabetic rats. Plasma lipid peroxide concentration was 2.5 times as high in diabetic rats, and erythrocyte GSH content 28% lower (Table 4). The administration of HT to diabetic rats reduced lipid peroxides in a dose-dependent manner (46% reduction at a dose of 0.5 mg kg−1 day−1, 80% reduction at a dose of 5 mg kg−1 day−1) whereas 10 mg kg−1 day−1 had a smaller effect than 5 mg kg−1 day−1 (68% inhibition compared to untreated diabetic rats) (Table 4). Erythrocyte GSH concentration increased after treatment with HT by 32–38% compared to untreated diabetic animals. There were no significant differences between groups in erythrocyte oxidized glutathione (Table 4).

Nitrosative stress, measured as serum 3-nitrotyrosine concentration, was clearly higher in diabetic rats than in normoglycemic animals (7.6-fold higher). The administration of HT to diabetic rats reduced 3-nitrotyroisine concentration in a dose-dependent manner by 37–86% (Table 4).

Plasma oxLDL concentration was 66% as high in diabetic rats as in normoglycemic animals (Fig. 2). Diabetic rats treated with HT had a lower plasma oxLDL concentration than untreated animals (32–47% reduction compared to untreated animals) (Fig. 2).

The variables for inflammation measured in this study showed statistically higher values in diabetic rats than non-diabetic animals (3-fold as high for MPOx and VCAM-1, and 1.3-fold as high for IL-1β) (Table 5). The administration of HT reduced MPOx by 32–66%, VCAM-1 by 50–81%, and IL-6 by 9–26% compared to untreated rats (Table 5).

Fig. 3 shows two representative examples of the morphological appearance of the aortic wall in a normoglycemic rats and in an untreated diabetic rats. The results of morphometric analyses in all groups are shown in Fig. 4. Vascular wall area was 33% greater in diabetic rats than in non-diabetic animals. Hydroxytyrosol reduced this area by 28–41% compared to untreated diabetic rats. The dose of 10 mg kg−1 day−1 had no significant effect on this variable. Vascular samples from diabetic rats had 42% more smooth muscle cells than in non-diabetic animals (Fig. 4). The administration of HT reduced this cell count by 12–19% compared to untreated diabetic animals.

4. Discussion

Our results show that the administration of HT modified some cardiovascular biomarkers in an experimental model of diabetes mellitus. We previously reported that the administration of extra-virgin olive oil modified some platelet and vascular variables in this experimental model [12]. Here we report the effect of HT, the main polyphenolic compound of virgin olive oil, in experimental diabetes.

One of the first biochemical processes leading to diabetic vasculopathy is oxidative stress due to chronic hyperglycemia [13] ; [14]. The results in our experimental model confirm the influence of oxidative stress, in light of the increased concentrations of lipid peroxides and reduced erythrocyte glutathione concentration (Table 4). Hydroxytyrosol modified oxidative stress through a reduction in lipid peroxides and an increase in gluthathione concentration. The antioxidant effect of HT has been widely described both in chemical and biological models [15] ; [16]. If oxidative stress is one of the most important initial steps in diabetic vascular disease, the inhibition of oxidative stress becomes an important effect to consider in explaining the beneficial effect of HT on endothelial dysfunction and in potentially preventing vasculopathy [17].

Respect to nitric oxide production diabetes mellitus increases peroxynitrite radicals and decreases constitutive nitric oxide production [18] ; [19]. Hydroxytyrosol did not significantly modify vascular nitric oxide production in our experimental model, but did reduce nitrosative stress (Table 3 ; Table 4) probably due to its antioxidant effect.

An important consequence of oxidative and nitrosative stress in diabetic vasculopathy is increased oxidation of lipoproteins such as LDL (oxLDL) [20]. The present results confirm that diabetic animals had higher plasma concentrations of oxLDL than normoglycemic animals. Hydroxytyrosol reduced the concentration of oxLDL in diabetic rats, a finding consistent with other reports that polyphenols in virgin olive oil prevent the oxidation of LDL in healthy humans and people with cardiovascular risk factors [21] ; [22]. The oxidation of LDL is a key step in the genesis and evolution of

arterial disease [23], so protecting LDL against oxidation is a potentially beneficial effect of efforts to prevent cardiovascular disease [24].

One aspect related to oxidative stress is the participation of inflammatory mediators and cytokines in the appearance of diabetic vascular disease. In the early stages of vasculopathy, increased leukocyte adhesion to the vascular endothelium results from increases in adhesive proteins such as VCAM-1. Myeloperoxidase also indicates specific activation of these leukocytes, and in this connection interleukins are known to participate in the development and evolution of vascular inflammation [25]. In the present study all three variables were higher in diabetic animals than in normoglycemic rats — results strongly suggestive of the appearance of inflammatory status in this experimental model (Table 5). Hydroxytyrosol significantly reduced these variables (Table 5). This effect has been previously described in experiments with induced cultured human umbilical vein endothelial cells [26]; [27] ; [28]. It was postulated that HT down-regulates nuclear factor NFκB, thereby decreasing the expression of these mediators. Moreover, a reduction in MPOx concentration of almost 75% was found in induced circulating neutrophils in rats [29], and reductions were also found in the intestinal wall in an inflammatory bowel disease model [30]. Regarding IL-1β production, HT and tyrosol were previously found to reduce interleukin-1β in rat brain slices in a hypoxia–reoxygenation model [31], a result probably due to the effect of the mechanism noted above on the down-regulation of nuclear factor NFκB [32]. Therefore HT may reduce vascular inflammatory status both in the early stages (leukocyte activation) and during the progression of vascular disease (IL-1β).

One of the main consequences of vascular inflammation is endothelial dysfunction, which also involves biochemical alterations in chronic hyperglycemia, mainly oxidative stress [3]. This endothelial dysfunction is represented by decrease in prostacyclin and nitric oxide production [33] and secondarily an increase in platelet function [33]. The present study demonstrates a deficit in prostacyclin and nitric oxide production together with increased platelet activity (platelet aggregation and thromboxane B2 production) in diabetic animals. This imbalance was offset, in part, by the administration of HT, which decreased platelet activity and platelet thromboxane B2 production, and also reduced, to a lesser extent, endothelial prostacyclin production. This profile was previously reported in samples of human blood [34] and blood from normoglycemic animals. These changes likely had an indirect effect on prostacyclin synthesis, since the antioxidant action of HT may curtail the negative role of free radicals in prostacyclin synthesis [8].

Overall, the administration of HT interfered with major biochemical processes leading to diabetic vasculopathy in the experimental model of diabetes mellitus tested here. These biochemical alterations led, among other consequences, to a proliferation of smooth muscle cells in the arteries, which in turn caused thickening of the vessel wall [35]. We also observed increases in the number of cells and the area of the arterial wall in diabetic animals compared to normoglycemic rats (Fig. 4). The administration of HT reduced both variables, and at the end of the 2-month experimental period the values for these variable were similar to those in non-diabetic animals.

Hydroxytyrosol was reported to inhibit the migration and proliferation of smooth muscle cells in vitro [36]. In rabbits fed a hyperlipidemic diet, oral supplementation

with HT led to a decrease in the area of aortic atherosclerotic lesions [37]. It has been postulated that HT may exert this effect by inhibiting smooth muscle cell ERK1/2 activation, and consequently blocking the cell cycle between the G1 and S phases [38]. However, HT may also exert this antiproliferative effect indirectly through an inhibitory action on mediators of oxidative stress and inflammation, which stimulate cell proliferation mechanisms [39]. This present study thus documents that HT can reduce the proliferation of muscle cells in the arterial wall in a model of experimental diabetes.

In conclusion, this study in rats with experimentally-induced diabetes mellitus shows that the administration of hydroxytyrosol influences the major biochemical processes leading to diabetic vasculopathy, and reduces cell proliferation in the vascular wall. Our results may have clinical applications in efforts to prevent diabetic vasculopathy, although additional studies will be needed to determine whether the incidence of vascular events decreases in patients with diabetes who take hydroxytyrosol daily.

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Figures Captions

Figure 1. Chromatographic profile of hydroxytyrosol purified after thermal treatment. The main compounds detected were hydroxytyrosol (99.6% purity) (peak number 1) and tyrosol (0.1% purity referred to dry matter) (peak number 2).

Figure 2. Plasma oxidized LDL concentration in non-diabetic rats (NDR) and diabetic rats treated with saline (DR) or with hydroxytyrosol for 2 months. aP<.0001 compared to NDR. bP<.05, cP<.01, dP<.0001 compared to DR. N=10 animals per group. eP<.05 with respect to all the other HT-treated groups.

Figure 3. Representative histological images of the aortic wall from nondiabetic rats, untreated diabetic rats (DR) and diabetic rats treated with hydroxytyrosol (HT). Hematoxylin and eosin staining, 10×. The images are representative of al least 70% of the samples.

Figure 4. Vascular wall area and smooth muscle cell count in non-diabetic rats (NDR) and diabetic rats treated with saline (DR) or hydroxytyrosol for 2 months. aP<.01 compared to NDR. bP<.05, cP<.01 compared to DR, dP<.01 with respect to all the other HT-treated groups. N=10 animals per group.

Table 1.

Mean values (mean ± SEM) of body weight, blood glucose and blood cellular counts in nondiabetic rats (NDR) and diabetic rats without treatment (DR) or treated with hydroxytyrosol (HT) (0.5, 1, 2.5, 5 and 10 mg kg^{-1} day^{-1} p.o.). N=10 rats per group

			DR				
	NDR	**DR**	**HT-0.5**	**HT 1.0**	**HT-2.5**	**HT-5.0**	**HT-10**
Body weight (g)	420±7	387±16	386±9	391±14	402±12	393±12	350 ±14
Blood glucose (mg/dl)	102±3[a]	491±8	507±7	512±9	487±28	474±14	496±5
Red blood cells (×10^{12}/L)	8.6±0.2	9.0±0.2	8.6±0.2	8.2±0.2	8.2±0.1	8.6±0.2	8.2±0.7
Leukocytes (×10^{6}/L)	6.1±0.3	6.4±1.9	5.9±0.5	5.3±0.4	5.4±0.6	5.0±0.3	5.1±0.6
Platelets (×10^{6}/L)	909±33	924±20	893±27	882±30	910±22	890±22	925±13
Hemoglobin (g/L)	16.0±0.4	16.7±0.5	15.8±0.3	16.3±0.3	16.6±0.2	16.7±0.3	16.4±0.5
Hematocrit (%)	46.8±1.0	46.4±1.3	44.3±0.8	45.8±0.8	48.5±0.7	48.4±0.7	45.2±1.2

a

$P<.0001$ with respect to all groups.

Table 2.

Mean values (mean ± SEM) of platelet variables maximum intensity of platelet aggregation (Imax) and platelet thromboxane B_2 (TxB_2) production in nondiabetic rats (NDR) and diabetic rats without treatment (DR) or treated with hydroxytyrosol (HT) (0.5, 1, 2.5, 5 and 10 mg kg^{-1} day^{-1} p.o.). *N*=10 rats per group

	Imax (ohms)	TxB_2 (pg/ml)
NDR	14.5±1.3	44.7±4.0
DR	28.3±1.4[a]	75.8±3.5[a]
DR + HT-0.5	26.3±0.8	67.2±3.2
DR + HT-1	21.7±0.4[b]	62.2±4.0[b]
DR + HT-2.5	18.6±0.8[b]	61.1±5.5[b]
DR + HT-5	16.8±1.1[b; c]	57.0±3.9[b; c]
DR + HT-10	10.8±0.9[b; c]	47.1±4.7[b; c]

a

P<.0001 with respect to NDR.

b

P<.0001 with respect to DR.

c

P<.05 with respect to HT-0.5, HT-1 and HT-2.5.

Table 3.

Mean values (mean ± SEM) of vascular variables: calcium-induced 6-keto-$PGF_{1\alpha}$ and nitrite + nitrate ($NO^-_2 + NO^-_3$) in nondiabetic rats (NDR) and diabetic rats without treatment (DR) or treated with hydroxytyrosol (HT) (0.5, 1, 2.5, 5 and 10 mg kg^{-1} day^{-1} p.o.). N=10 rats per group

	6-keto-$PGF_{1\alpha}$ (pg/mg aorta)	$NO^-_2 + NO^-_3$ (nmol/mg aorta)
NDR	0.9±0.1	12.0±1.3
DR	0.4±0.03[a]	6.6±0.5[b]
HT-0.5	0.4±0.03	7.4±0.7
DR + HT-1	0.5±0.07	7.5±0.3
DR + HT-2.5	0.9±0.06[c; d]	7.2±0.5
DR + HT-5	0.9±0.08[c; d]	6.5±0.2
DR + HT-10	0.8±0.09[c; d]	10.1±0.8[c; e]

a

P<.05,

b

P<.0001, with respect to NDR.

c

P<.05, with respect to DR.

d

P<.05 with respect to HT-05 and HT-1.

e

P<.05 with respect to HT-0.5, HT-1, HT-2.5 and HT-5.

Table 4.

Mean values (mean ± SEM) of oxidative stress (thiobarbituric acid reactive substances –TBARS- and reduced –GSH- and oxidized –GSSG- glutathione) and nitrosative (3-nitrotyrosine) stress variables in nondiabetic rats (NDR) and diabetic rats without treatment (DR) or treated with hydroxytyrosol (HT) (0.5, 1, 2.5, 5 and 10 mg kg^{-1} day^{-1} p.o.). N=10 rats per group

	TBARS (nmol/mg protein)	GSH (µmol/g Hb)	GSSG (µmol/g Hb)	%GSSG with respect to GSH + GSSG	3-nitrotyrosine (pg/ml)
NDR	0.24±0.03	102±22	38.9±8.4	27.2±3.6	0.79±0.02
DR	0.61±0.05[b]	73.3±6.2[a]	29.8±3.5	29.0±1.6	6.10±0.90[b]
DR+ HT-0.5	0.33±0.04[d]	97.3±4.7	44.4±6.7	30.4±3.5	3.85±0.48
DR+ HT-1	0.28±0.03[d]	90.1±4.9	33.3±5.4[f]	26.7±3.5	2.43±0.19[d]
DR+ HT-2.5	0.15±0.02[d ; e]	106±3.8[c]	29.4±4.3	27.0±2.4	0.97±0.04[d ; e]
DR+ HT-5	0.12±0.05[d ; e]	101±5.2[c]	43.4±7.1	29.2±3.3	0.93±0.36[d ; e]
DR+ HT-10	0.19±0.02[d ; e]	89.9±8.9	42.6±3.5	32.6±3.4	0.85±0.12[c ; e]

a

P<.05;

b

P<.0001, with respect to NDR.

c

P<.05;

d

P<.0001, with respect to DR;

e

P<.05 with respect to HT-0.5 and HT-1;

f

P<.05 with respect to HT-0.5; HT-5 and HT-10.

Table 5.

Mean values (mean ± SEM) of inflammatory variables: myeloperoxidase (MPOx), VCAM-1 and interleukin-1β (IL-1β) in nondiabetic rats (NDR) and diabetic rats without treatment (DR) or treated with hydroxytyrosol (HT) (0.5, 1, 2.5, 5 and 10 mg kg^{-1} day^{-1} p.o.). N=10 rats per group

	MPOx (ng/ml)	VCAM-1 (ng/ml)	IL-1β (pg/ml
NDR	4.08±0.23	0.37±0.03	1.8±0.05
DR	12.30±1.23[a]	1.16±0.09 [a]	2.3±0.06[b]
DR + HT-0.5	4.28±0.56[d]	0.22±0.41[d ; f]	2.1±0.006
DR + HT-1	4.13±0.48[d]	0.43±0.06[d]	2.0±0.05
DR + HT-2.5	7.11±0.83[d ; e]	0.48±0.06[d]	1.9±0.05
DR + HT-5	7.14±0.77[c ; e]	0.58±0.06[d]	1.7±0.06[d]
DR + HT-10	8.29±0.99[c ; e]	0.50±0.04[d]	1.7±0.1[d]

a

$P<.0001$;

b

$P<.002$, with respect to NDR;

c

$P<.01$;

d

$P<.0001$, with respect to DR;

e

$P<.05$ with respect to HT0.5 and HT-1;
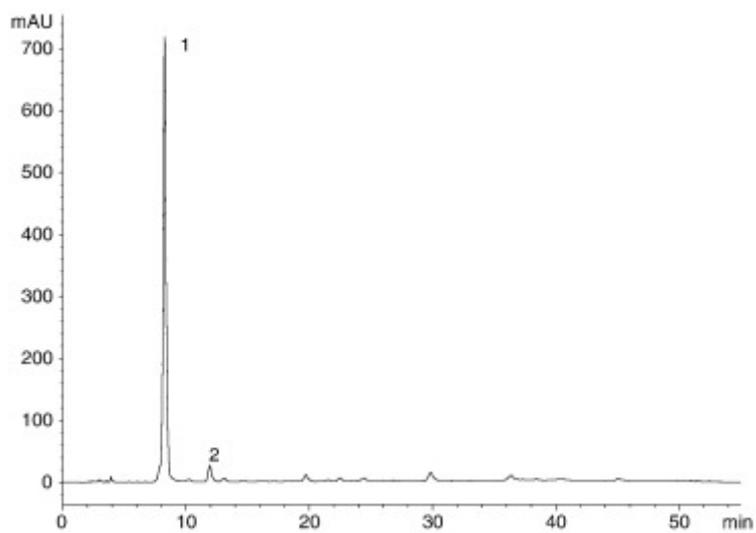
f

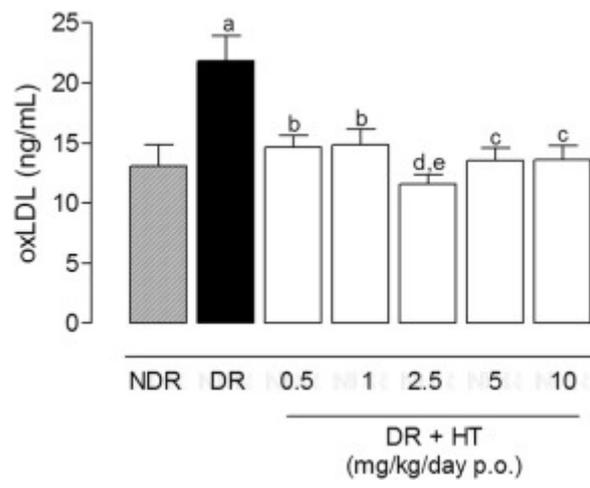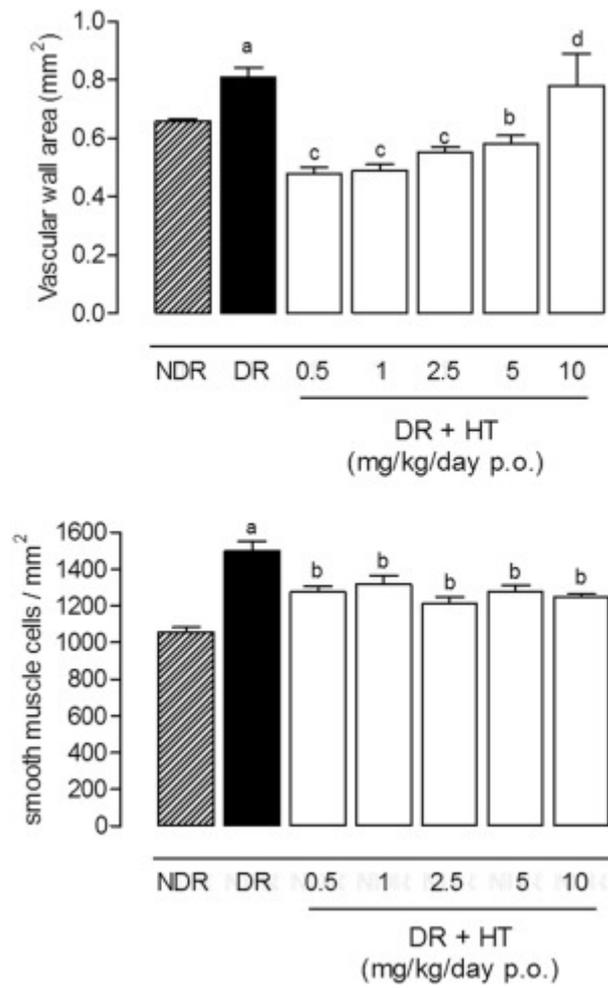$P<.05$ with respect to all the other HT-treated groups.

Figure 1

Figure 2

Figure 3

Figure 4

Nondiabetic rat   Diabetic rat (DR)   DR + HT 0,5 mg/kg/day   DR + HT 1 mg/kg/day

DR + HT 2,5 mg/kg/day   DR + HT 5 mg/kg/day   DR + HT 10 mg/kg/day