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# Ferulic acid, a bioactive component of rice bran, improves oxidative stress and mitochondrial biogenesis and dynamics in mice and in human mononuclear cells☆,☆☆

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**Abstract**

The aim of the study was to characterize the vascular effects of rice bran enzymatic extract (RBEE). ApoE−/− mice were fed a high-fat/cholesterol diet (HFD) or HFD supplemented with 5% RBEE for 21 weeks. RBEE prevented development of atherosclerotic plaques and oxidative stress in mouse aorta as well as the down-regulation of markers of mitochondrial biogenesis. Analysis of the bioactive components identified ferulic acid (FA) as responsible component. In healthy human volunteers, FA intake reduced NADPH oxidase activity, superoxide release, apoptosis and necrosis in peripheral blood mononuclear cells. Differentiation and proliferation of endothelial progenitor cells were improved. In summary, the study identifies FA as a major active component of rice bran, which improves expression of mitochondrial biogenesis and dynamics markers and reduces oxidative stress in a mouse model of vascular damage as well as in endothelial cells and human mononuclear cells.
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*Keywords:* Rice bran enzymatic extract; Ferulic acid; Atherosclerosis; Mitochondrial function; Oxidative stress

## Chemical compounds

Ferulic acid (PubChem CID: 445858)
γ-Oryzanol (PubChem CID: 51346127)
β-Sitosterol (PubChem CID: 222284)
γ-Tocotrienol (PubChem CID: 53394606)

## 1. Introduction

Vascular mitochondria are involved in vascular pathologies such as atherosclerosis, and mitochondrial function is crucial for the development and severity of atherosclerotic plaques [1–6]. Since mitochondria are both sources and targets of reactive oxygen species (ROS), mitochondrial ROS contribute to atherogenesis by inducing oxidative modifications of lipoproteins, endothelial dysfunction, vascular inflammation, thrombosis and smooth muscle cell proliferation [7]. Generation of mitochondrial ROS also leads to oxidative modifications of mitochondrial proteins and lipids and to mutations in the mitochondrial DNA [2–4]. The amount of mitochondrial damage correlates with the extent of atherosclerosis in both mice and man [1–6].

Mitochondrial structure and function are preserved by tightly regulated processes called mitochondrial biogenesis and dynamics [8]. Peroxisome proliferative activated receptor-α coactivator 1 (PGC-1α) is a master regulator of mitochondrial biogenesis [9,10] and expression of genes coding for the subunits of the respiratory chain and other proteins [10]. PGC-1α activity is controlled though posttranscriptional modifications by metabolic sensors such as sirtuin 1 (SIRT1) or AMP-activated protein kinase (AMPK) to adapt mitochondrial biogenesis to energy expenditure [11,12]. Mitochondrial dynamics is the regulation of mitochondrial morphology by the dynamic processes of fusion and fission. For example, fusion is mediated by mitofusin proteins, and fission is regulated by dynamin and fission proteins [13].

Mitochondria and oxidative stress regulate the critical balance of cellular survival, regeneration and apoptosis in the vascular system. Under pathological conditions of increased endothelial apoptosis,

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regeneration of injured endothelium is enhanced by circulating endothelial progenitor cells (EPCs) [14]. EPCs are inversely correlated with atherosclerosis risk factors and adverse clinical outcomes and therefore represent integrative biomarkers of vascular health [15].

Cardiovascular effects of natural dietary supplements such as rice bran are of growing interest [16]. Rice bran is a byproduct of rice milling with lipid-lowering, antioxidant, antidiabetic and anti-inflammatory activities due to the high content in phytochemicals such as γ-oryzanol (OZ) [esters of ferulic acid (FA) and of triterpene alcohols or plant sterols], phytosterols and tocotrienols [17]. The use of rice bran by the food industry has been limited by the poor solubility of its components and by fast lipase degradation leading to rancidity. These weaknesses were overcome by the enzymatic extraction [18]. Previous studies demonstrated that rice bran enzymatic extract (RBEE) prevents atherosclerosis and protects from endothelial dysfunction by increasing cellular stress resistance [19,20]. Although the chemical properties of rice bran are known, the main components responsible for the effects, the mechanisms of action and its relevance for human biology remain unclear. We hypothesized that mitochondrial function is a crucial mediator of the effects of RBEE.

The aims of this study were to characterize the effects of RBEE on mitochondrial biogenesis and dynamics in relation to vascular oxidative stress in ApoE−/− mice. We identified FA as the main compound present in the extract responsible for the actions observed. We measured FA and FA-derived metabolites accumulation in mice liver and kidney and its human bioavailability. Finally, we performed a human study evaluating the impact of FA consumption on peripheral blood mononuclear and endothelial progenitor cells.

## 2. Material and methods

### 2.1. Rice bran enzymatic extract

RBEE was prepared and chemically characterized as previously described [18]. Briefly, raw rice bran was introduced in a bioreactor, pH (8.0) and temperature (60°C) controlled. The extraction was performed with a hydrolytic trypsin- and chymotrypsin-like endoproteases mixture (Bioproteasa LA450; Biocon Española, Spain). Nutraceutical composition in RBEE is as follows: OZ (8950 mg/kg), phytosterols (3553 mg/kg), tocotrienols (170 mg/kg) and tocopherols (93.4 mg/kg).

### 2.2. Animals and diets

All animal experiments were performed conforming the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes and were approved by the Ethic Committee for Animal Experimentation of the University of Seville (Spain) (AGL2013-407791-P). Male apolipoprotein E knockout (ApoE−/−) mice on a C57BL/6J background and wild-type (C57BL/6J) mice were purchased from Charles River Laboratories (L'Abresle, France). At 7 weeks of age, ApoE−/− mice were randomized to high-fat diet (HFD) (TD 88137; Teklad, Envigo, Madison, WI, USA) containing 0.15% (w/w) cholesterol and 42% (% kcal) fat or HFD supplemented with 5% (w/w) of RBEE (HFD 5% RBEE) for 21 weeks. As nonatherosclerotic control, age-matched C57BL/6J wild-type mice were kept on standard diet (STD) (2014; Teklad, Envigo, Madison, USA). At 28 weeks of age, mice were sedated by an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg) and sacrificed by exsanguination.

### 2.3. Cell culture

Bovine aortic endothelial cells (BAECs; passages 3–6) were grown at 37°C in a 5% $CO_2$ atmosphere in Dulbecco's modified Eagle medium supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin; Life Technologies), 4 mM L-glutamine and 10% fetal bovine serum (Gibco, Invitrogen) [21]. Cells were harvested for 24 h and then cultured for 18 h in the presence of 367.6 mg/l RBEE or one of its main bioactive components at the same concentration at which those are found in the dose of RBEE used: 5.5 μM FA, 5.5 μM OZ, 1.5 μM β-sitosterol (β-Sitos) or 0.12 μM γ-tocopherol. Then, cells were co-incubated with 100 μM $H_2O_2$ for 4 additional hours, washed twice, lysed by sonication in the suitable buffer for the different assays performed and frozen at −80°C until analyzed.

### 2.4. Human study: serum values

All human studies have been approved by the local ethics committee (number 162/15) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. All subjects provided written informed consent prior to their inclusion in the study. Healthy middle-aged adults following a balanced diet were included in the study. The exclusion criteria were as follows: active smoking, age out of the range 25–50 years, being under any medical treatment, taking dietary supplements, following a restricted diet or recent digestive illness.

From the mice study, the human equivalent dose was calculated (151.88 mg FA per day for a mean human weight of 70 kg) as described by Sharma and McNeill [22]. Due to the short-term nature of this pilot study, the five volunteers included in the study (3 males and 2 females, mean age: 35.4±3.7 years) consumed the higher dose of 500 mg FA per day as recommended by the manufacturer (Source Naturals, Scotts Valley, USA) for antioxidant support prior to breakfast and followed a balanced diet. On the first day, FA absorption was measured at the following times: 0 min, 30 min, 60 min, 180 min, 360 min and 24 h. An additional tube was collected before FA consumption for serum lipid, glucose and liver enzymes measurement.

### 2.5. Human study: isolation and treatment of peripheral blood mononuclear cells (PBMCs)

The effects of FA were tested *ex vivo* in cultured PBMCs from five untreated healthy volunteers. For each subject, 1 million freshly isolated PBMCs were plated in RPMI medium at 37°C in 5% $CO_2$ atmosphere and treated with 5.5 μM FA or an equal volume of solvent (control condition) for 18 h, starting after the isolation. Then, the cells were co-treated with 100 μM $H_2O_2$ alone or in the presence of FA for 4 additional hours to induce oxidative stress and apoptosis. Furthermore, to test the *in vivo* effects of FA in humans, PBMCs were isolated from 40 ml of blood before and after 15 days of treatment with FA by Ficoll density gradient centrifugation [21,23,24]. PBMCs from the *in vivo* study were directly treated with solvent or 100 μM $H_2O_2$ for 4 h after the isolation, washed twice and used for the assays.

### 2.6. Determination of glutathione levels, aconitase activity and lipid peroxidation

Spectrophotometric measurement of reduced (GSH) and oxidized (GSSG) forms of glutathione was based on 5,5-dithio-bis-(2-nitrobenzoic acid) consumption and measured in aortic tissue from mice and BAECs as previously described [25]. Results are presented as GSH/GSSG ratio. Aconitase catalyzes the reaction of sodium citrate to isocitrate using $NADP^+$ as electron acceptor. Aconitase activity from aortic homogenates was measured spectrophotometrically by registering the formation of NADPH as previously described [26] and is expressed as mU per mg protein. Malondialdehyde (MDA) quantification served as an indicator of lipid peroxidation and was carried out in aortic tissue and BAECs using the ALDetect Lipid Peroxidation Assay Kit following the manufacturer's recommendations. Results are expressed as nM MDA per mg protein [25].

### 2.7. NADPH oxidase (NADPHox) activity assay

NADPHox is the main source of ROS in mononuclear blood cells. A lucigenin-enhanced luminescence assay was performed to determine basal and phorbol myristate acetate (PMA)-stimulated NADPHox activity in human PBMC from the *in vivo* study as reported before [23,24].

### 2.8. Quantification of atherosclerotic plaques in the aorta

Three-micrometer cross sections of 4% paraformaldehyde-fixed aortic arches were used to quantify atherosclerotic lesions after hematoxylin/eosin staining. Tissue recorded with an Olympus BX61 microscope under the same magnification (10× objective) and atherosclerotic plaque area was measured using ImageJ v1.45 software.

### 2.9. Western blot

Immunoblots were carried out in aortic tissue and BAEC protein lysates as previously described [19] with the following primary antibodies: anti-peroxiredoxin III (1:500; Biomol-Enzo Life Sciences, Lörrach, Germany), anti-peroxiredoxin-$SO_3$ (1:500; AbFrontier, Seoul, Korea), anti-peroxiredoxin-I (1:500; Cell Signaling Technology, Beverly, MA, USA) anti-AMPKα2 (1:1000; Santa Cruz Biotechnology, Heidelberg, Germany), anti-p-AMPKα (Thr172) (1:1000, Cell Signaling Technology), LC3A (1:500, Cell Signaling Technology), p62 (1:750, Santa Cruz Biotechnology), Beclin-1 (1:500, Cell Signaling Technology), GAPDH (1:10,000, Santa Cruz Biotechnology) or anti-β-actin (1:1000, Santa Cruz Biotechnology). Horseradish-peroxidase-conjugated goat anti-rabbit IgG (1:4000) or donkey anti-goat IgG (1:4000) was used against the appropriate species of primary antibody. Immune complexes were visualized with the use of enhanced chemiluminescence and exposition to autoradiography films (GE Healthcare, Munich, Germany), which were developed in an Agfa Curix 60 system. Densitometric analyses of the resulting bands were performed using Image Studio Lite v.4.0.21 software (LI-COR, Lincoln, NE, USA), and β-actin was used as internal control to verify equal protein loading in all blots.

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