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Aristotelia chilensis, rutin and quercetin amielorates acute vascular endothelial dysfunction in rat thoracic aorta exposed to oxidative stress

[*Aristotelia chilensis*, rutina y quercetina alivia la disfunción endothelial aguda en aorta torácica de rata expuesta a estrés oxidativo]

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Abstract: The role of endothelial dysfunction (ED) and excessive oxidative stress in the development of cardiovascular diseases has recently been highlighted. The present study examined the effect of a hydro-ethanolic extract of a Chilean berry *Aristotelia chilensis* (folk name "maqui), and its flavonoids antioxidants rutin (RT) and quercetin (QC), on the responsivity of blood vessels exposed to oxidative stress. For functional relaxation studies, the isolated rat aortic rings (RARs) of male Wistar rats were used. To model acute oxidative stress *in vitro*, RARs were incubated in Krebs' solution containing either high glucose (46 mM) or the O2- generator pyrogallol (50 µM). RARs exposed to either glucose or pyrogallol displayed attenuated maximum vasorelaxation responses to the endothelium-dependent vasodilator acetylcholine (Ach), and reduced nitric oxide (NO) bioavailability. These effects were fully suppressed by pre-incubation of the vessels with the maqui berry extract (MBE), RT and QC. Both, removal of the endothelium and the addition of nitric oxide synthase (NOS) inhibitor, NG-Nitro-L-Arginine Methyl Ester (L-NAME) increased the phenylephrine (Phe) response. These observation suggest that MBE, QC and RT may protect against high glucose and pyrogallol-induced endothelial dysfunction via enhanced the generation and bioavailability of NO.

Keywords: *Aristotelia chilensis*, oxidative stress, endothelial dysfunction

Resumen: Últimamente, el rol de la disfunción endotelial y el estrés oxidativo en el desarrollo de enfermedades cardiovasculares ha adquirido un importante foco de atención. El presente estudio examinó el efecto de un extracto hidroalcohólico de frutos de *Aristotelia chilensis* (nombre vulgar: maqui) y su flavonoides antioxidantes rutina y quercetina sobre la capacidad de respuesta de los vasos sanguíneos expuestos a estrés oxidativo. Para este estudio se utilizaron anillos de aorta aislados de ratas Wistar macho. Los anillos se incubaron en solución Krebs con alta glucosa (46 mM) o con el generador de radical superóxido pirogalol (50 µM) para generar el estrés oxidativo agudo *in vitro*. Aortas expuestas a glucosa o pirogalol exhibieron una significativa disminución de la respuesta vasorelajante dependiente del endotelio cuando se estimulan con acetilcolina, reduciendo significativamente la biodisponibilidad de óxido nítrico. Dicho fenómeno fue revertido cuando los anillos se pre-incubaron tanto, con el extracto como con los flavonoides rutina y quercetina. La eliminación del endotelio y la presencia de inhibidor de la óxido nítrico sintasa (NG - nitro-L - arginina metil éster, L-NAME) aumentó la respuesta de la fenilefrina. Los hallazgos en este estudio sugieren que el extracto de maqui, quercetina y rutina pueden evitar la disfunción endotelial generada por alta glucosa y pirogalol posiblemente debido a su potente capacidad antioxidante que permite una mayor producción o biodisponibilidad de óxido nítrico.

Palabras clave: *Aristotelia chilensis*, estrés oxidative, disfunción endothelial.

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INTRODUCTION

Oxidative stress refers to a condition in which cells are subject to the presence of a large amount of oxygen molecules and chemical derivatives termed reactive oxygen species (ROS) (Loscalzo, 2003). Clinical and experimental investigations show that oxidative stress plays a major role in the pathogenesis and development of complications of both types of diabetes mellitus (DM). However, the exact mechanism by which oxidative stress contributes to and accelerates the development of complications in DM remains to be clarified. In patients with diabetes oxidative stress induces abnormalities in endothelial cell function by altering the synthesis or degradation of NO, which is one of the central features of endothelial dysfunction (ED) (Karunakaran & Park, 2013). ED is a systemic pathological state which can be broadly defined as an imbalance between vasodilatory and vasoconstricting compounds produced by the endothelium (Deanfield, 2005). NO is produced by many cells in the body. However, its production by vascular endothelium is particularly important in the regulation of blood flow. ED is primarily due to reduction in NO bioavailabilty which is, accordingly, a marker for vascular health (Kolluru *et al.*, 2012). ED may arise from and/or contribute to a diversity of disease processes, including DM, hypercholesterolemia,hypertension, and environmental toxicities, such as smoking tobacco products and air pollution (Cai & Harrison, 2000).

Hyperglycemia is a major source of ROS. Because antioxidants attenuate damage to injured tissues caused by free radicals, it has been proposed that diabetic individuals may benefit from dietary antioxidant supplementation. Recent clinical studies report that antioxidants reverse ED by enhancing endogenous NO-mediated vasorelaxation (Zamora & Villamena, 2013). The molecular mechanisms underlying this effect are not clearly understood.

Many studies show that consuming a diet rich in antioxidant-containing foods is associated with a reduced risk of cardiovascular disease (Cespedes *et al.*, 2008; Slavin & Lloyd, 2012). *Aristotelia chilensis* (Molina) Stuntz (Elaeocarpaceae) an evergreen shrub that grows up to 4 m tall. It is native to the Patagonia region of Chile and Argentina. Mature maqui berries are the size of large blueberries with a deep purple or black color. MB is one of the highest sources of antioxidants (Céspedes *et al.*, 2010; Rubilar *et al.*, 2011). Significant health benefits have been observed in persons that consume maqui (Seeram, 2008). We've recently provided *in vitro* and *in vivo* evidence that MBE directly normalizes DE, glycemia and lipidemia in diabetic rats (Fuentes *et al.*, 2013). However, further evidence is required to link compounds that are active in MBE with mechanisms that underlie DM pathogenesis.

We propose that MBE and its flavonoids RT and QC, restores vascular function through enhanced bioavaibility of endothelium-derived nitric oxide during oxidative stress. Rutin, a flavonol glycoside comprising quercetin and the disaccharide rutinose, that is found in higher plants, has numerous health benefits including neuroprotection (Nakayama *et al.*, 2011), attenuated renal inflammation and apoptosis (Arjumand *et al.*, 2011), reduced risk of cancer (Lin *et al.*, 2012), coronary heart disease (Fernandes *et al.*, 2010) and hyperglycemia, and improved ED (Fuentes *et al.*, 2013). To test this hypothesis we measured the effects of the maqui berry extract (MBE), RT and QC on the Ach-mediated vasorelaxation in control RARs and RARs exposed to either 46 mM glucose or 50 µM pyrogallol (a superoxide generator). Prolonged exposure to high glucose *in vitro* or *in vivo* generates reactive oxygen species (Rodriguez-Manas *et al.*, 2003; Patel *et al.*, 2013)], impairs the bioactivity of NO and inhibits acetylcholine Ach-induced endothelium-dependent relaxation (Qian *et al.*, 2006) Pyrogallol, a catechin compound, is an O_2^- generator used to investigate the role of ROS in biological systems (Kim *et al.*, 2008).

MATERIAL AND METHODS

Plant material

The fruits of *Aristotelia chilensis*, maqui, matured in summer, were collected from Rinconada, a golf course adjacent to the Cato River in the central valley of Provincia de Ñuble, Biobío Region, Chile, with their identification confirmed by Dr. Victor Finot of the Department of Animal Production, Faculty of Agronomy, University of Concepción, Chile. A voucher specimen has been deposited in the herbarium of the Department of Basic Sciences of University of Bio-Bio, Chillán, Chile.

An aqueous ethanol extract was prepared by adding EtOH-H_2O (6:4) to 430 g of dried and ground pulp of the MB. After maceration, the extract was filtered on Whatman paper, and the solvent was evaporated under reduced pressure (50° C) in a rotary evaporator. The crude hydro-alcoholic extract is a dark purple residue (MBE, yield 32 g) that may be stored at 2-4° C until use.

Phytochemical profile

Using HPLC-DAD-ESI-MS and -NMR hyphenated procedures, the following compounds were identified in MBE: p-coumaric acid, rutin and a mixture of catechin and epi-catechin; a mixture of p-coumaric acid and p-hydroxybenzoic acid; gentisic acid, sinapic acid and procyanidin B; gallic acid, quercetin, myricetin, delphinidin-3-glucoside and cyanidin-3-glucoside; cyanidin-3-O-glucoside, 4-hydroxybenzoic acid, ferulic acid, a mixture of cyanidin and catechin; delphinidin-3-glucoside, delphinidin-3,5-diglucoside, delphinidin-3-sambubioside and cyanidin-3-sambubioside; a mixture of proanthocyanidins (probably trimers and tetramers) and a dimer identified as proanthocyanidins B, together with cyanidin-3-sambubioside-5-glucoside and delphinidin-3-sambubioside-5-glucoside, and finally were isolated trimers and tetramers of proanthocyanidins unidentified, and was isolated a mixture of free sugars (Cespedes *et al.*, 2010). All compounds were identified by comparison of their retention times in the HPLC with those from library HPLC data. A subset of structures were confirmed by comparing their spectral features (UV, IR, NMR, and GC/MS) with a data base, with values reported in the literature, and by comparison with known control samples (Cespedes *et al.*, 2009; Cespedes *et al.*, 2010).

The anthocyanin composition of MB was determined by means of HPLC, UV, and GC/MS analysis. The HPLC chromatogram of the anthocyanins extract revealed that all peaks correspond to different pigments that co-elute indicating the presence of eight different anthocyanins that were identified and quantified. Compounds identified by comparison of HPLC retention times, GC/MS, NMR and photodiode array (DAD/UV/VIS) spectroscopic data analysis. (Cespedes *et al.*, 2010) include quercetin, myricetin, and the aglicone of rutin and ferulic acid, together with several other anthocyanins.

Experimental Animals

Wistar male rats (8-11 months) weighing in the range of 180-250 g were used for the study. The animals were obtained from central animal house, Departmento de Ciencias Básicas, Universidad del Bio-Bío, Chillán, Chile, and were housed in an air conditioned colony room at 23 ± 4° C with 12h light and 12 h dark cycles. The experimental animals were given food and water *ad libitum*. Standard pellets obtained from Purina Lab Chow were used as a basal diet during the experimental period. The study was performed under the supervision of the Ethical Committee of the Biobío University in Chillán, Chile and in conformity with "The Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996).

Isolated thoracic aorta preparation

Male Wistar rats weighing 180-210 g were anesthetized with diethyl ether and killed by exsanguination. The thoracic aorta was immediately excised and placed in a Kreb's solution of the following composition (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1 and carefully cleaned from adhering fat and connective tissue. The aorta was cut into transverse rings (3–4 mm), which were then mounted on triangular stirrups and suspended under 2 g resting tension in organ baths (Radnoti, Monrovia Ca, USA) containing 50 ml Kreb's solution and maintained at 37° C, pH 7.4, gassed with 95% O_2 and 5% CO_2. Isometric measurements were recorded with a transducer (Radnoti) and displayed on Power Lab software (AD Instruments, USA). The tissue was allowed to equilibrate for 60 min before conducting the experiments, during which time the resting tension was readjusted to 2 g as required. The aortic rings were submaximally contracted with 1 μM phenylephrine (Phe). The functional presence of endothelium was verified by the ability of 1 μM Ach to induce relaxation, and an endothelium-denuded rings, or 100 μM L-NMA preparation was used to confirm the involvement of NO with sodium nitroprusside (SNP, 10 nM). RARs were incubated for 20 min in Krebs-Henseleit solution to which was added MBE (500 μg/ml), QC (10 μM) or RT (50 μM) or its vehicle (DMSO). The contractile responses of RARs were expressed in percentages of change (delta) with respect to their maximum tension in 1 μM Phe. Vascular responses to the vasodilator Ach were reported in the percentage of reduction in tension (remaining contraction) compared to the tone level induced by contraction in Phe. To evaluate the effect of pyrogallol on RARs, pyrogallol (1-100 μM) and Phe (1 μM) were used to induce steady contraction in endothelium-intact aortic rings. Ach (1 μM) was then added to induce relaxation. The half-maximum effective concentration (EC_{50}), which was defined as the concentration of pyrogallol that induced 50% of maximum inhibition of relaxation by Ach after the contraction elicited by Phe, was calculated (50 μM) using Origin 6.0 software.

Similarly, glucose concentrations from 5 mM to 100 mM, were used to define the EC 50 (46 mM). The second series of experiments were designed to evaluate the protective effects of MBE, RT and QC against on endothelial dysfunction in RARs induced by high glucose and pyrogallol. Before addition of MBE, RT and QC, the incubation time of RARs with pyrogallol and high glucose, were 20 and 120 min respectively.

Statistical analysis

The experimental results (usually 2 - 4 from each animal) were averaged and used in subsequent analyses. Results are expressed as the mean ± S.E.M. Student's t-test was used to analyze the significance of the results. The values of $P < 0.01$ were considered significant.

Chemical and solvents

All enzymatic reagents were from DiaSys Diagnostic Systems GmbH (Germany). Rutin, Quercetin, Phenylephrine, Acetylcholine chloride, NG-nitro-L-arginine methyl ester (L-NAME), sodium nitroprusside (SNP), dimethyl sulfoxide (DMSO) and all the other reagents and compounds used for Kreb's solution were purchased from Sigma (St. Louis, MO, USA).

RESULTS

Effect of NG-Nitro-L-Arginine Methyl Ester and absence of endothelium on Phe contraction of RARs incubated with pyrogallol and high glucose

To assess the contribution of endothelium-derived NO in response to Phe after incubation with pyrogallol and high glucose, pre-contracted aortas were denuded of endothelium or exposed to a NO synthase inhibitor L-NAME. Figure 1 depicts the effect of 50 µM pyrogallol and 46 mM glucose on values of contractile response to 1µM Phe in endothelium-intact, endothelium-denuded, and L-NAME-treated aortic rings incubated in Krebs–Henseleit solution.

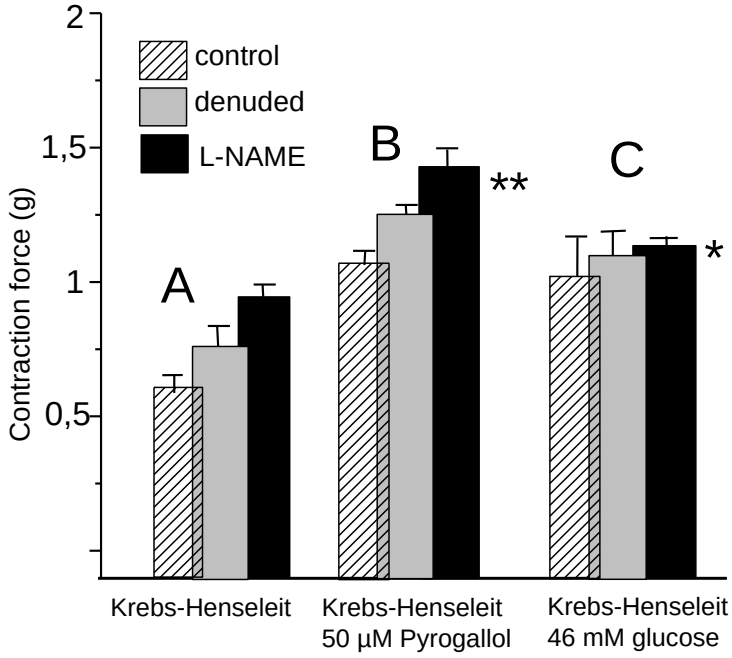

Figure 1

Stimulation of rat aortic ring with 1µM phenylephrine. Assays were done with normal medium (group A), with normal medium containing 50 µM pyrogallol (group B) and normal medium with 46 mM glucose (group C). All three groups of assay have denuded, intact (control of group) and 100µM L-NAME-incubated rings. The data represent the mean ± SEM (n = 8 animals). **P < 0.001 compared with A group; *P < 0.01 compared with A group. In group A, with normal medium, L-NAME addition and functional removal of the endothelium caused a significant Phe-induced contraction of the rings. In group B and C, incubated with 50 µM pyrogallol and 46 mM glucose respectively, phenylephrine displayed more potent contraction effects than group A.

Effect of MBE, RT and QC on Phe contraction of RARs incubated with pyrogallol and high glucose

Addition of MBE, RT and QC resulted in a greater inhibition of Phe-contraction of RARs preincubated with of 50 µM pyrogallol or 46 mM glucose as compared with control values contraction. This is shown in Figure 2

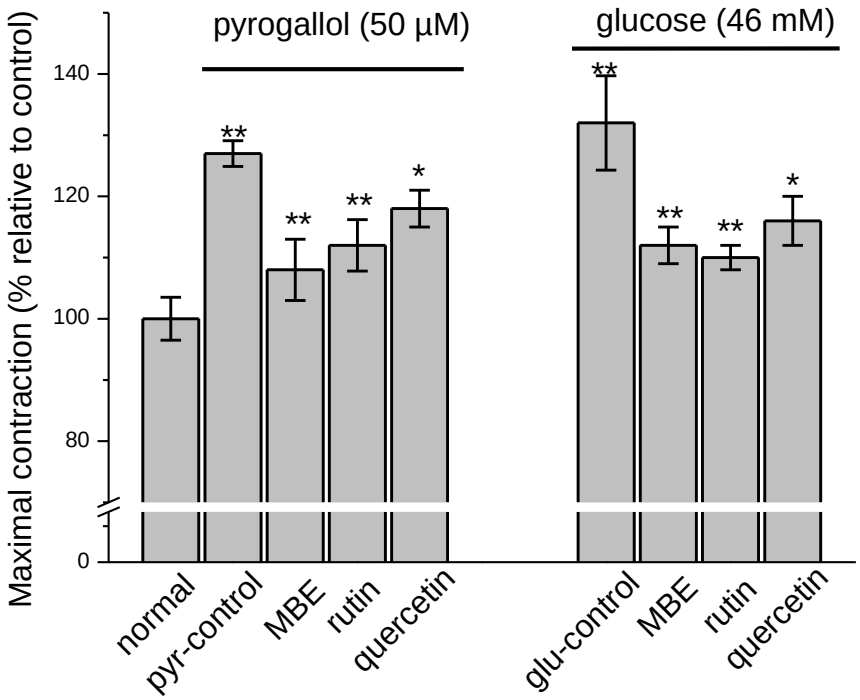

Figure 2
Stimulation of intact aortic rings with 1µM Phe. Assays were done with normal medium (control), with normal medium containing 50 µM pyrogallol and normal medium with 46 mM glucose. Rings were incubated in the presence of MBE, RT or QC. The data represent the mean ± SEM (n = 7 animals). $^{++}P < 0.001$ compared with control; $^{**}P < 0.001$ compared with pyr-control ; $^{*}P < 0.01$ compared with A group.

Effect of MBE, RT and QC on Ach relaxation of precontracted RARs incubated with pyrogallol and high glucose

Ach-mediated relaxation in the aortic rings of rats depends on NO synthesis (Cachofeiro & Nasjletti, 1991). In the present study, reactivity to Ach in Phe precontracted aortas after 20 min incubation with pyrogallol (Figure 3) and high glucose (Figure 4) revealed significantly ($P < 0.001$) decreased endothelium-dependent relaxation compared to control. However, Ach-induced relaxation of the aortic rings treated either with MBE, QC or RT was significantly greater ($P < 0.001$; 0.001 and 0.01 respectively) compared to aortas exposed to pyrogallol or high glucose alone. Furthermore, MBE, RT and QC did not alter isometric tension of aortic rings in basal pre-constricted conditions.

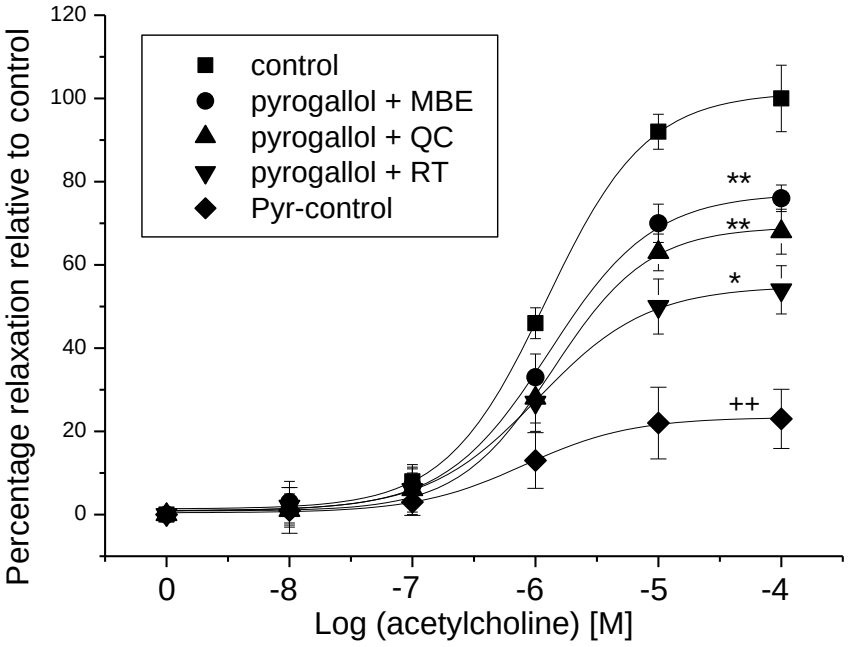

Figure 3
Stimulation of intact aortic rings with 0-1^{-4}M Ach. Assays were done with normal medium (control), with normal medium containing 50 µM pyrogallol and pyrogallol-medium with MBE, RT or QC. The data represent the mean ± SEM (n = 7 animals). $^{++}$*P*<0.001 compared with control; ***P*<0.001 compared with pyr-control ; **P*<0.01 compared with pyr-control.

Relaxation was expressed as the percentage change from Phe-contracted levels. Treatment with the extract, RT or QC 20 min after pyrogallol incubation was effective in suppressing the pyrogallol-induced ED. Similar results were observed in RARs incubated with 46 mM glucose for 120 min (Figure 4).

Incubation with high glucose for 120 min markedly disminished the NO formation in RARs as reflected by the reduction of relaxation with Ach when compared with control. Co-treatment with MBE (500 µg/ml), rutin (50 µM) or quercetin (10 µM) reduced the high glucose-inhibition of NO rise.

DISCUSSION

Endothelial dysfunction in the setting of cardiovascular risk factors such as hypercholesterolemia, diabetes mellitus, chronic smoking and hypertension is dependent in part on the production of ROS and the subsequent decrease in vascular bioavailability of nitric oxide (NO) (Cespedes *et al.*, 2008; Rochette *et al.*, 2013).

The present study aimed to test whether MBE (ED_{50} = 60 µg/ml), QC (10 µM) or RT (50 µM) have a beneficial effect against hyperglycemic injury and pyrogallol-induced oxidative stress on isolated RARs,and to investigate the possible mechanism(s). Quercetin and rutin are widely distributed in edible fruits and vegetables. QC is the major flavonoid in the human diet with a mean daily intake estimated to be 50–500 mg (Deschner *et al.*, 1991). The choice of 10 µM QC in this study is based on previous studies in which this concentration was found to induce endothelium-dependent and NO-dependent (against Phe-induced contraction), and endothelium-independent (against high K+-induced contraction), relaxation in isolated euglycemic and diabetic rat aortas (Ajay *et al.*, 2006). The choice of 50 µM RT is further based on studies that used RARs pre-contracted with Phe, with or without endothelium (Xia *et al.*, 2005, Fuentes *et al.*, 2013).

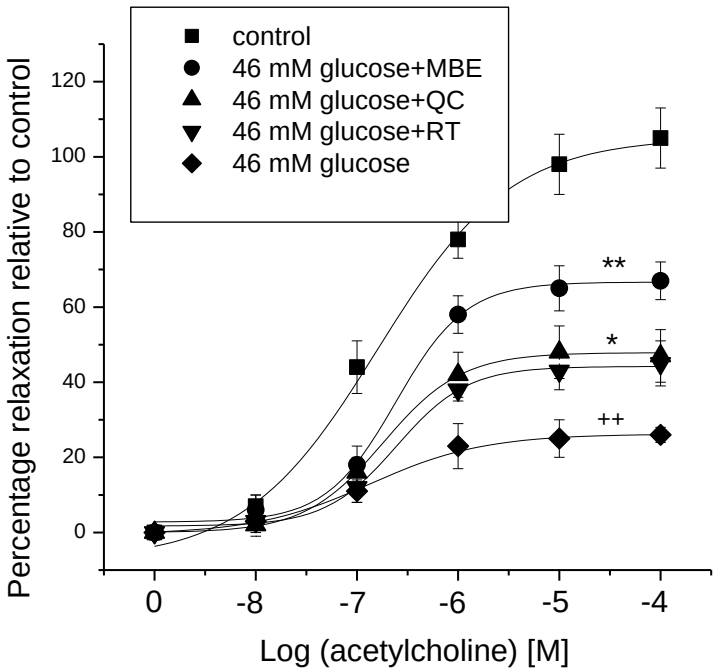

Figure 4
Stimulation of intact aortic rings with 0-1^{-4}M Ach . Assays were done with normal medium (control) and with normal medium containing 46 mM glucose with either MBE, RT or QC. The data represent the mean ± SEM (n = 7 animals). ++*P* < 0.001 compared with control; ***P* < 0.001 compared with 46 mM glucose alone; **P* < 0.01 compared 46 mM glucose alone.

The increment in the contractile responses after Phe treatment in denuded and L-NAME treated aortic rings (Figure 1A) is, at least in part, due to the absence of basal NO bioavailability generated by these condition (Dora *et al.*, 2000). Enhanced negative endothelial modulation by NO as a compensatory mechanism to slow the development of tension produced by Phe has been reported (Padilha *et al.*, 2008). This data is supported by our observations in endothelium-denuded rings of diabetic rats, where the Phe-induced contraction is greater than in the intact rings, similar when the rings were incubated in the presence of L-NAME (Fuentes *et al.*, 2013). RARs incubated with pyrogallol (B) and high glucose (C) showed significantly higher sensitivity to Phe compared to the untreated rings. Increased maximal responses to Phe in aortic rings after incubation with pyrogallol and glucose may be due to an augmented superoxide anion liberation/generation, which inactivates the residual NO and counteracts NO-mediated negative modulation even in the presence of high NO production and antioxidant defense (Davel *et al.*, 2006). Augmented production of the superoxide anion by pyrogallol and high glucose may lead to decreased tissue bioavailability of NO via a facile radical/radical reaction that occurs more rapidly than the reaction of with superoxide dismutase (Pacher *et al.*, 2007). This phenomenon alters endothelial regulation of vasomotion in a variety of disease conditions. Although O_2^- has been reported to scavenge NO within the vascular wall to reduce its biological half-life, its direct relevance to attenuated endothelium-derived vasodilatation reamins to be determined (Pechánová & Simko, 2007).

MBE, QC and RT produced significant reductions in contractile response to phenylephrine of vascular smooth muscle of RARs incubated with high glucose and pyrogallol. This reduction appears to correlate with the increase of NO bioavailability following MBE and flavonoids treatment. The mechanisms responsible for an increased contractile response of arteries to vasoconstrictor agonists are not completely elucidated, although alterations of endothelial function, enhanced calcium mobilization and inhibition of Na+/K+-ATPase activity appear to contribute to changes in vascular reactivity (Xavier *et al.*, 2003). Similarly, stimulation of smooth muscle cells with α_1-adrenoceptors agonists increases NO synthesis (Dora *et al.*, 2000).

The stimulated NO production in control RARs in response to ACh as revealed in Figs 3 and 4 was significantly lower after high glucose and pyrogallol incubation. However, acute exposure to MBE, RT or QC effectively reversed the glucose and pyrogallol-induced impairment of endothelium-dependent relaxations, thus indicating that the anti-oxidative (reduction of ROS) activity of MBE, RT and QC helps to preserve the bioavailability of NO. This finding is further supported by the improvement of endothelial function observed in isolated diabetic aortas that has been given acute and chronic treatment of maqui berries extract (Fuentes *et al.*, 2013).

The present study, for the first time, suggest that the beneficial effects of MBE, QC and RT intake arise from a reduction in the effects of superoxide and other that reactive oxygen species, in diabetes, possibly through their effects on the ability of the endothelium to synthesize, release or respond to endogenous NO or NO donated by nitro-vasodilators.

CONCLUSIONS

Our findings suggest that that maqui berry extract, quercetin and rutin protect against high glucose and pyrogallol-induced endothelial dysfunction most probably by potent antioxidant properties (Céspedes *et al.*, 2010) including an increase in NO production and enhanced bioavailability of NO. Novel pharmacological approaches that promote endothelium-derived nitric oxide synthesis, and that target vascular ROS-producing enzymes, may open new avenues toward retarding the progression of diabetes associated vascular complications.

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