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Research Article
Dose-dependent Differential Mechanism of Quercetin-induced Vasodilatations in Isolated Perfused Rat Mesenteric Vascular Bed

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Abstract

Epidemiological studies indicate that low incidence of cardiovascular disease is associated with dietary intake of polyphenolic compounds, which are abundantly present in fruits and vegetables. There is solid evidence that quercetin, a polyphenolic compound, exerts vasodilator effects in addition to its antioxidant activity. Therefore, in this study, the contribution of shear stress-induced nitric oxide to the vasodilator effect of quercetin in mesenteric bed was investigated. Dose-dependent vasodilator effects of quercetin on the perfusion pressure increased by phenylephrine were recorded in the presence of L-arginine/cGMP pathway inhibitors or superoxide dismutase in the perfused mesenteric vascular beds isolated from rats. Quercetin (1, 5 and 10 μM) concentration-dependently decreased the perfusion pressure raised by phenylephrine (3-6 μM) in the endothelium-intact mesenteric bed. The relaxations occured at 1 and 5 μM quercetin were significantly inhibited by nitric oxide synthase inhibitor, N_{ω}-nitro-L-arginine (L-NA,100 μM) or the guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM), while relaxations to 10 μM quercetin were not affected. Removal of endothelium significantly reduced the relaxations at lower concentrations of quercetin but was without effect on relaxations induced by 10 μM. Calmidazolium (0.5 μM), a calmodulin inhibitor did not significantly affect the quercetin responses but a superoxide anion scavanger, superoxide dismutase (SOD, 100 U mL^{-1}) significantly improved the quercetin-induced relaxations especially at 1 and 5 μM. These findings suggest that quercetin induces endothelium-dependent vasodilatations at lower concentrations by increasing the bioactivity of sustained nitric oxide release evoked by perfusion pressure. However, the vasodilatations induced by high concentrations of quercetin are endothelium-independent.

Key words: Quercetin, rat mesenteric bed, perfusion pressure, endothelial nitric oxide, vasodilatation

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Flavonoids constitute a large class of polyphenols abundantly found in plants and their products. This group has several sub-classes such as flavonols, flavones and flavanols. Epidemiological studies indicate that low incidence of cardiovascular disease is associated with dietary intake of polyphenolic compounds (Renaud and de Lorgeril, 1992; Hertog *et al*., 1993; Scalbert *et al*., 2005a, b). Several beneficial effects of these compounds have also been reported from the experimental investigations, which support the epidemiological studies. Data from the experimental studies showed the inhibitor effect of these compounds on proliferation and migration of vascular smooth muscle cells (Iijima *et al*., 2000, 2002) and antihypertensive effects in spontaneously hypertensive rats (Duarte *et al*., 2001). There are also many studies showed the antiaggregant (Gryglewski *et al*., 1987), antioxidant (Rice-Evans *et al*., 1996; Heijnen *et al*., 2001; Benito *et al*., 2002; Ertug *et al*., 2010, 2013) and vasodilator effects of polyphenolic compounds (Fitzpatrick *et al*., 1993; Chen and Pace-Asciak, 1996; Andriambeloson *et al*., 1997; Flesch *et al*., 1998; Naderali *et al*., 2000; Abeywardena *et al*., 2002; Zenebe *et al*., 2003). It is well known that endothelium plays an important role in the vascular smooth muscle relaxation through the generation of Nitric Oxide (NO), which can be decreased or abolished in the presence of Nitric Oxide Synthase (NOS) inhibitors such as N_{ω}-nitro-L-arginine (L-NA) and N_{ω}-nitro-L-arginine methyl ester (L-NAME) (Furchgott and Zawadzki, 1980; Palmer *et al*., 1987; Moncada *et al*., 1991). It has been suggested that quercetin, a major polyphenolic compound and most abundant flavonoids stimulate relaxation of vascular smooth muscle in coronary arteries (Cogolludo *et al*., 2007). In aortic rings, quercetin-induced relaxation was found to be due to enhancement of NO levels and thus activation of NOS associated with increase in endothelial intracellular calcium (Kubota *et al*., 2001). In addition, this flavonoid scavenges oxygen free radicals, resulting enhancement of nitric oxide production. Thus, beneficial effects of quercetin at the vascular levels can be explained by the increase in the NO-cGMP production (Benito *et al*., 2002). However, it has been reported that quercetin also relaxed smooth muscle endothelium independently in rat conductance and resistance vessels (Duarte *et al*., 1993; Chen and Pace-Asciak, 1996; Perez-Vizcaino *et al*., 2002; Ajay *et al*., 2006). On the other hand, it was reported that quercetin-induced vasodilation was reduced by NOS inhibitors or SK channel inhibitor apamin but not by BK or IK channel inhibitor charybdotoxin in rat thoracic aorta (Nishida and Satoh, 2009) and that quercetin-induced vasodilatation, sensitive to gap-junction inhibitor is endothelium dependent and mediated by EDHF in rat mesenteric arteries (Nishida and Satoh, 2013). It appears that the vasodilator action mechanism of quercetin is quite complex in vascular tissues and not well studied in mesenteric vascular bed. Thus, the vasorelaxant effect of this flavonoid in the mesenteric vascular bed can be a useful model for vasoactive drugs, which are yet to be investigated in detail.

On the basis of these considerations, in the present study, it was investigated the mechanism of quercetin-induced vasodilatation and possible contribution of endothelial nitric oxide in these relaxations in the isolated perfused rat mesenteric vascular bed.

MATERIALS AND METHODS

Animals: Male Wistar rats weighing 200-250 g used throughout the experiments were kept under standard laboratory conditions (12 h light/dark). The experimental procedures were approved by the animal care committee of the University of Çukurova (DETAUM) and the studies were carried out in accordance with the principles of laboratory animal care (National Institutes of Health guideline; publication No. 86-23, revised 1984).

Isolated rat mesenteric bed perfusion: Male Wistar rats (200-250 g) were anaesthetized with halothane, then exsanguinated and the abdominal cavity of individual rats was opened and an incision was made as reported previously (Secilmis *et al*., 2007). The mesenteric vascular bed was dissected away from the connecting tissues immediately and transferred to a jacketed organ bath, where it was perfused with the bubbled (95% O_2 and 5% CO_2) Krebs-Henseleit solution maintained at 37°C throughout the experiments. A peristaltic pump (Buchler Instruments, Lenexa, KS, USA) was used to obtain the constant perfusion flow rate of 6.5 mL min^{-1}. The perfusion pressure of the mesenteric bed was continuously measured with a pressure transducer (Keller PRC 21K-10) and recorded on a polygraph (Gemini 7070). Changes in the perfussion pressure induced by any drug represented the alterations in vascular resistance.

Experimental protocol: All experiments were performed in the presence of indomethacin (1 μM), a cyclooxygenase inhibitor. After a 60 min equilibrium period of the tissue, perfusion pressure was increased by addition of phenylephrine (3-6 μM) to the perfusion fluid. When the perfussion pressure reached the steady state, acethylcholine (0.01-0.1 μg/0.1 mL) was applied as a close-arterial bolus

injection to each tissue in order to test the endothelial function. In these studies, the preparations less or no responsive to phenylephrine or acetylcholine were discarded.

In the first series of experiments, optimal doses of quercetin (1, 5 and 10 μM) determined in the preliminary experiments were added to the perfusion fluid to induce vasodilatation in the isolated rat mesenteric vascular beds precontracted by phenylephrine (3-6 μM). When the concentration-response curves to quercetin were achieved, the mesenteric bed was washed with fresh Krebs solution to return to the basal state. After the incubation period of 60 min, the first series of responses to phenyleprine and quercetin were obtained again. This group was accepted as a control. In the other groups, the effects of NO synthase inhibitor, L-NA (100 μM), the guanylate cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 5 μM), a nitric oxide scavenger, hydroxocobalamin (HC, 100 μM), a calmodulin inhibitor, calmidazolium (0.5 μM) and a superoxide anion scavanger, superoxide dismutase (SOD, 100 U mL^{-1}) were investigated on the quercetin-induced relaxations in the isolated perfused rat mesenteric vascular beds. Agents were applied in the following method; L-NA, ODQ or calmidazolium were added to the perfusion fluid 30 min before the second series of the experiments, while SOD was added with the first dose of quercetin (1 μM) to the perfusion fluid after the 60 min incubation period. The HC was administered just after obtaining the maximum relaxant response to 10 μM quercetin in the second series of the experiments.

Quercetin responses were also investigated in the endothelium-denuded rat mesenteric artery to assess the contribution of endothelium to quercetin-induced relaxations. Removal of endothelium was achieved by saponin (50 mg L^{-1}) perfusion for 10 min, followed by equlibration in normal Krebs solution for 60 min before treatment with test agents. Precontracted tissues with phenylephrine have less or no relaxant response to acetylcholine accepted as endothelium-denuded preparations.

Drugs and solutions: All drugs were prepared on the day used in the experiments. Indomethacin, phenlyephrine hydrochloride, acethylcholine chloride, quercetin, N_{ω}-nitro-L-arginine, calmidazolium, hydroxocobalamin and superoxide dismutase (SOD) were all purchased from Sigma Chemical Company (St., Lois, MO, USA) and saponin from Fluka Chemie AG (Deisenhofen, Germany). Indomethacin, quercetin and calmidazolium were dissolved in dimethyl sulfoxide (DMSO). The final DMSO concentration was 0.01% when it was added to 1 L perfusion reservoir and this did not affect the changes of the pressure induced by phenylephrine, acetylcholine or quercetin. Hydroxocobalamin, N_{ω}-nitro-L-arginine, SOD and saponin were dissolved in Krebs solution. Phenylephrine, hydrochloride and acethylcholine chloride were dissolved in distilled water.

Statistical analysis: All values are expressed as Mean±SEM. The increase in perfusion pressure induced by phenylephrine was expressed as mmHg. The vasodilatation in mesenteric vascular beds were measured as a percent decrease in the perfusion pressure elevated by phenylephrine. Paired Student's t-test was used to compare the perfusion pressures. The p values less than 0.05 were considered statistically significant from control.

RESULTS

Effect of quercetin on the pressure increased by phenylephrine and effect of NOS inhibition on the vasodilatation induced by quercetin in the isolated perfused rat mesenteric vascular bed: In the control experiments, basal perfusion pressure was 19.80±2.0 mmHg. Continuous perfusion of tissue with phenylephrine (3-6 μM) increased the pressure to 68.90±5.6 mmHg. Addition of quercetin in the range of 1-10 μM to the perfusion fluid decreased the pressure concentration-dependently. In the perfusion pressure, dose-dependent decrease due to vasorelaxations induced by quercetin (1, 5 and 10 μM) were 21.89±1.25, 52.82±2.67 and 72.29±2.06%, respectively. The quercetin-induced relaxations at all concentrations were not significantly affected by 1 μM indomethacin, a cyclooxygenase inhibitor (Data not shown). However, in the presence of indomethacin (1 μM) plus L-NA (100 μM), vasodilatations induced by quercetin at concentrations 1 and 5 μM were significantly reduced, while relaxations to 10 μM quercetin were not affected. Dose-dependent quercetin responses in the presence of L-NA were 1.25±0.31, 21.14±3.59 and 69.06±4.15%, respectively (Fig. 1).

Effect of guanylyl cyclase inhibition by ODQ on the quercetin-induced vasodilatation in the isolated perfused rat mesenteric vascular bed: The vasorelaxations induced by low concentrations of quercetin were reduced significantly by ODQ (5 μM). Quercetin-induced relaxations at 1 and 5 μM concentrations reduced to 1.33±0.42 and 14.79±2.45% from 15.52±2.73 and 38.92±2.23%, respectively. At the high concentration of quercetin (10 μM), ODQ did not affect the relaxation. Values were 72.34±6.23% for quercetin control and 67.68±5.95% for quercetin in the presence of ODQ (Fig. 2).

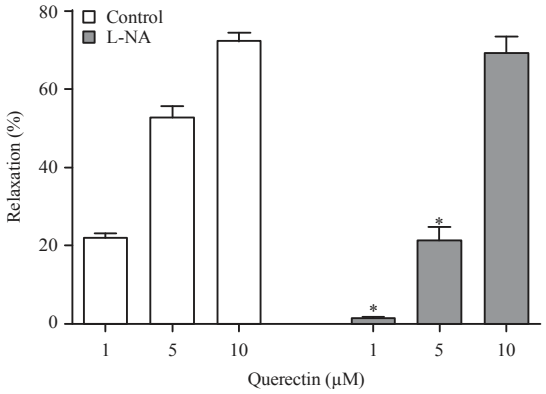

Fig. 1: Relaxant responses to quercetin (1, 5 and 10 μM) and effect of L-NA (100 μM) on the quercetin-induced relaxations in the isolated perfused rat mesenteric bed pressure increased by phenylephrine (3-6 μM). The decrease of the perfusion pressure was expressed as a percentage of the rise in the pressure induced by phenylephrine (n = 8). *Indicates the statistical significance compared to control group ($p<0.05$)

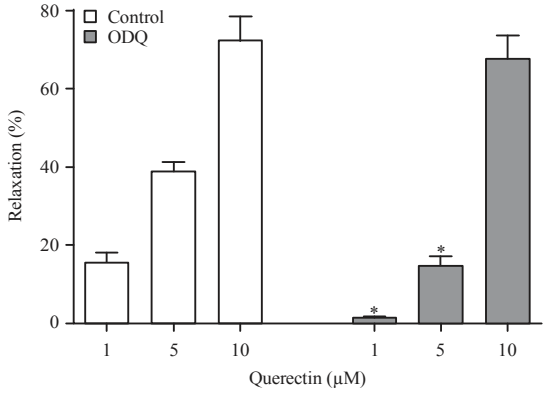

Fig. 2: Effect of ODQ (5 μM) on the relaxant responses induced by quercetin (1, 5 and 10 μM) on the pressure increased by phenylephrine (3-6 μM) in the isolated perfused rat mesenteric bed. The decrease of the perfusion pressure was expressed as a percentage of the rise in the pressure induced by phenylephrine (n = 6). *Indicates the statistical significance compared to control group ($p<0.05$)

Effect of endothelium removal on the vasodilatation induced by quercetin in the isolated perfused rat mesenteric vascular bed: Saponin perfusion (50 mg L^{-1}, for 10 min) was used to remove the vascular endothelium of the mesenteric vascular bed. After saponin perfusion, vasodilator response to quercetin concentrations at 1 and 5 μM were significantly reduced to 3.03±2.01 and 28.03±2.31% from 14.96±2.44 and 48.20±4.59%, respectively. However,

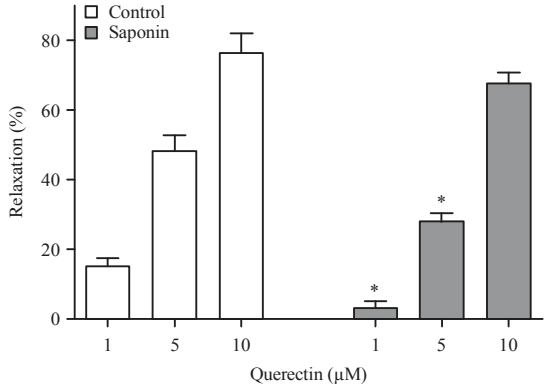

Fig. 3: Effect of endothelium denudation with saponin (50 mg L^{-1}) on the relaxant responses induced by quercetin (1, 5 and 10 μM) on the pressure increased by phenylephrine (3-6 μM) in the isolated perfused rat mesenteric bed. The decrease of the perfusion pressure was expressed as a percentage of the rise in the pressure induced by phenylephrine (n = 5). *Indicates the statistical significance compared to control group ($p<0.05$)

removal of endothelium did not significantly affect the relaxant response of quercetin at 10 μM. Values for 10 μM quercetin-induced relaxations in endothelium-intact and denuded tissues were 76.48±5.62 and 67.69±2.97%, respectively (Fig. 3).

Effect of hydroxocobalamin on the vasodilatation induced by quercetin in the endothelium-intact and denuded isolated perfused rat mesenteric vascular bed: Quercetin-induced relaxations at all concentrations observed in the presence of L-NA were completely reversed by 100 μM hydroxocobalamin in the endothelium-intact rat mesenteric vascular bed. However, this effect was also observed in the endothelium-denuded mesenteric bed (Data not shown).

Effect of inhibition of Ca^{2+}-calmodulin coupling by calmidazolium on the vasodilatation induced by quercetin in the isolated perfused rat mesenteric vascular bed: The relaxations induced by quercetin were not significantly affected by 0.5 μM calmidazolium. Values for 1, 5 and 10 μM quercetin-induced relaxations in control group were 16.18±2.07, 38.34±2.15 and 65.76±5.08% and in calmidazolium-treated groups were 11.57±1.87, 30.89±4.05 and 69.84± 7.0%, respectively (Fig. 4).

Effect of SOD on the vasodilatation induced by quercetin in the isolated perfused rat mesenteric vascular bed: Vasodilator responses-induced by quercetin (1, 5 and 10 μM)

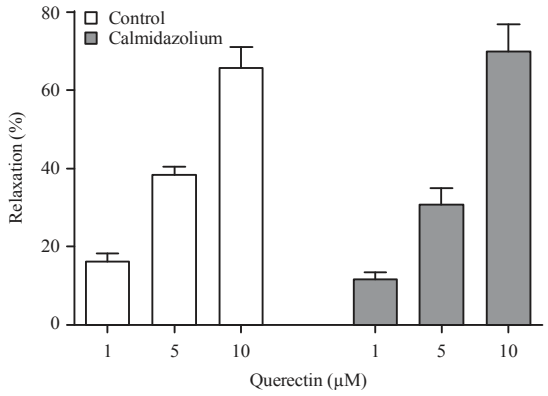

Fig. 4: Effect of calmidazolium (0.5 μM) on the relaxant responses induced by quercetin (1, 5 and 10 μM) on the pressure increased by phenylephrine (3-6 μM) in the isolated perfused rat mesenteric bed. The decrease of the perfusion pressure was expressed as a percentage of the rise in the pressure induced by phenylephrine (n = 6)

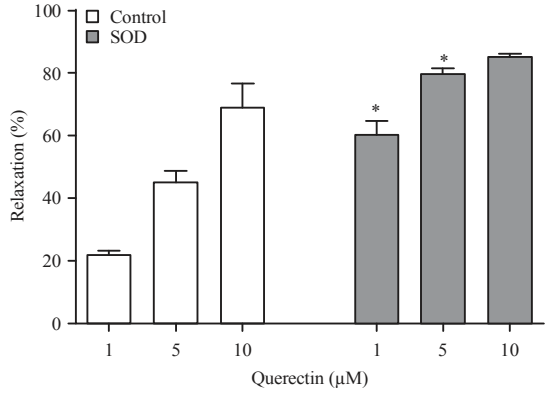

Fig. 5: Effect of SOD (100 U mL^{-1}) on the relaxant responses induced by quercetin (1, 5 and 10 μM) on the pressure increased by phenylephrine (3-6 μM) in the isolated perfused rat mesenteric bed. The decrease of the perfusion pressure were expressed as a percentage of the rise in the pressure induced by phenylephrine (n = 5). *Indicates the statistical significance compared to control group ($p<0.05$)

were significantly increased by the presence of SOD especially with lower concentrations of quercetin (1 and 5 μM). Relaxations were 21.7±1.29 and 45.08±3.63% in control groups, while values for 1 and 5 μM quercetin-induced relaxations in the presence of SOD were increased to 60.27±4.46 and 79.49±18.66%, respectively. Relaxations at the highest concentration of quercetin (10 μM) were not significantly changed in the presence of SOD. Relaxation values for quercetin alone and in the presence of SOD were 68.68±7.81 and 84.9±1.00%, respectively (Fig. 5).

DISCUSSION

In this study, it was investigated that vasodilator effect of quercetin on the perfusion pressure increased by an α_1-adrenergic receptor agonist phenylephrine and whether nitric oxide is contributed to the quercetin-induced vasorelaxations in the perfused mesenteric vascular beds isolated from rats. It was found that this polyphenolic compound leads to both endothelium-dependent and independent relaxation in a dose-dependent manner.

It is known that there is a wide range of polyphenols, which have desirable biological properties, such as protective effect on cardiovascular system including antithrombotic, antioxidant, anti-ischemic and vasorelaxant effect for human being (Scalbert *et al*., 2005a; Jasuja *et al*., 2012; Liu *et al*., 2014). Some polyphenolic compounds have endothelium-dependent relaxant activity on the vascular smooth muscle (Zenebe *et al*., 2003). In the present study, perfusion of mesenteric vascular bed with cumulative concentrations of quercetin decreased the pressure raised by phenylephrine. It is well known that vascular endothelium has an important role in the regulation of vascular smooth muscle tone. Endothelium-dependent relaxation induced by chemical agents, such as acetylcholine, bradykinin is mediated by endothelium derived relaxing factor (Furchgott and Zawadzki, 1980) identified as nitric oxide (Palmer *et al*., 1987), which is synthesized from L-arginine in the endothelial cells (Palmer *et al*., 1988). This labile molecule leads to relaxation of vascular smooth muscle, inhibition of platelet aggregation and adhession (Moncada *et al*., 1991).

Biosynhtesis of nitric oxide is selectively inhibited by Nitric Oxide Synthase (NOS) enzyme inhibitors such as L-arginine analogue, N_ω-nitro-L-arginine (L-NA). It has been reported that relaxations of vascular smooth muscle induced by quercetin was endothelium-dependent and mediated by nitric oxide release, which was abolished by NOS inhibition or endothelium denudation in the rat aortic rings (Kubota *et al*., 2001). In this study carried out in the mesenteric vascular bed, relaxations induced by lower concentrations (1 and 5 μM) of quercetin were inhibited by NOS inhibitor L-NA, while higher concentration of quercetin (10 μM)-induced relaxant response was not affected. In addition to other mechanism, this phenomenon indicates a role for endothelial NO in mediating relaxant responses to quercetin.

These findings are consistent with the previous studies, showing that quercetin-induced relaxation has two

components, mediated by nitric oxide and non-nitric oxide pathway in the rat aorta (Chen and Pace-Asciak, 1996; Fusi *et al*., 2003). The NO increases intracelllular cGMP levels by activation of soluble guanylate cyclase (Schrammel *et al*., 1996). In the present study, vascular bed was perfused with ODQ in order to examine, whether L-NA sensitive component of relaxation induced by quercetin is coupled to cGMP production. Perfusion of the bed with soluble guanylyl cyclase inhibitor ODQ inhibited the relaxations induced by this flavonoids at lower concentrations, while relaxation at 10 μM quercetin was not affected. Similarly, quercetin-induced relaxations were decreased in the absence of endothelium in lower concentrations, while those at high concentration were not affected. Because endothelial NOS activity was present only in the endothelium of the vascular tissue, these findings are consistent with those in the presence of selective inhibitors of NOS or soluble guanylyl cyclase.

These results suggest that vasodilator responses to quercetin in lower concentrations, at least partially were involved in NO/cGMP pathway. In the vascular bed, synthesis and release of endothelial NO can be stimulated by some chemical agonists in a Ca^{2+}-calmodulin dependent manner (Busse and Mulsch, 1990; Adeagbo *et al*., 1994). However, activation of eNOS and production of NO induced by mechanical forces (isometric contraction, pressure and shear stress acting on the endothelium) have shown to be independent of sustained increase in the calcium levels and insensitive to calcium-calmodulin inhibitor (Fleming *et al*., 1998; Secilmis *et al*., 2014). It has been reported that quercetin-induced endothelial nitric oxide release was due to the activation of NOS. This was associated with increase in the endothelial intracellular calcium level in the thoracic aorta (Kubota *et al*., 2001). In the present study, effect of calcium-calmodulin inhibitor calmidazolium was tested on the quercetin-induced relaxation to evaluate the contribution of intracellular Ca^{2+} increase in vascular endothelial cells. Vasodilator responses induced by quercetin at lower concentration, sensitive to inhibitors of L-arginine/cGMP pathway were not affected by the presence of calmidazolium.

These findings suggest that calcium-calmodulin coupling was not involved in the quercetin-induced relaxations in the mesenteric bed. Therefore, quercetin-induced relaxations may arise from, at least in part, increasing the bioactivity of Ca^{2+}-independent sustained NO release in response to phenylephrine-induced increase in perfusion pressure. It has been suggested that flavonoids increase NOS activity and subsequent NO production (Benito *et al*., 2002). It was also shown that quercetin decreased NADPH oxidase-induced O_{2-} levels and increased NOS activity in spontaneously hypertensive rats (Sanchez *et al*., 2006). In another study, it has also been reported that quercetin act as a protective agent in the penile erectile tissue, in which this flavonoids protected the exogenous NO from superoxide anions and increased NO bioavailability (Ertug *et al*., 2010).

Similar effects were observed in mouse gastric fundus, in which inhibitor effects of superoxide anion generators on nitrergic relaxations were reversed by the flavonoids (Ertug *et al*., 2013). In the present study, quercetin was also applied in the presence of SOD to test antioxidant activity of the flavonoids. It was also observed that quercetin-induced relexations were increased in the presence of SOD and this effect was marked especially at the lower doses of quercetin. Taken together, these findings suggest that quercetin may have an antioxidant effect at low concentrations. Residuel relaxant responses to some stimuli, such as acetylcholine might be contributed by NO even in the presence of optimal NOS inhibition, which is further inhibited by NO scavengers, indicating activity of NOS inhibitors-insensitive NO (Simonsen *et al*., 1999; Chauhan *et al*., 2003). In the previous study, it was observed that the generation of residual NO is also stimulated by vasoconstriction in mesenteric bed, in which NO scavenger hydroxocobalamin augmented the phenylephrine-induced increase in the pressure in the presence of NOS inhibitors (Secilmis *et al*., 2014).

Therefore, in order to exclude this possibility, the mesenteric bed was perfused with hydroxocobolamin after the maximal relaxation was achieved by 10 μM quercetin in the presence of L-NA. A dramatic reversal of relaxation has been observed in the presence of NOS inhibitor. This finding raised the possibility that NO is a major component of the quercetin-induced relaxation by the contribution of NOS inhibitors-insensitive NO. In the present study, however, quercetin-induced relaxation was reversed by NO scavenging with hydroxocobolamin similarly to that of endothelium intact beds. Considering endothelial NOS activity was present only in the endothelium of the vascular tissue, reversal of quercetin-induced relaxations by hydroxocobalamin in absence of endothelium, this effect points out posibility of chemical interaction between quercetin and hydroxocobolamin, resulting scavenging of the flavonoids.

CONCLUSION

Quercetin can cause two types of relaxations, endothelium-dependent, which is sensitive to inhibitors of L-arginine/NO/cGMP pathway and independent, both of them produced in a concentration dependent manner. The former took place at lower concentrations. However,

the latter occured at higher concentrations in the isolated perfused rat mesenteric bed.

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