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Original

Quercetin and resveratrol ameliorate nickel-mediated hypercontraction in isolated Wistar rat aorta

Shahnawaz Ahmad WANI, Luqman Ahmad KHAN, Seemi Farhat BASIR

Department of Biosciences, Faculty of Natural Science, Jamia Millia Islamia, New Delhi 110025, India

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Abstract

Purpose: The ameliorative potential of quercetin and resveratrol on isolated endothelium-intact aortic rings incubated with nickel was examined.

Method: The effect of varying concentrations of quercetin and resveratrol was investigated on isolated Wistar rat aortic rings using an organ bath system over vasoconstrictor phenylephrine (PE) at 1 μM. To delineate the mechanism of action, isolated aortic rings were pre-incubated with pharmacological modulators, such as verapamil 1 μM, apocynin 100 μM, indomethacin 100 μM or N-G-nitro-L-arginine methyl ester (L-NAME) 100 μM, separately, before incubation with 100 μM quercetin and 30 μM resveratrol. To assess the ameliorative and prophylactic potentials of quercetin and resveratrol, aortic rings were also incubated with quercetin or resveratrol for 40 min, followed by incubation with nickel for 40 min.

Results: At 100 μM, quercetin caused 29% inhibition of contraction, while resveratrol at 30 μM caused 55% inhibition of contraction in aortic rings compared with control. Aortic rings incubated with contractile modulators, such as verapamil, apocynin, indomethacin or N-G-nitro-L-arginine methyl ester (L-NAME), along with quercetin or resveratrol at their concentrations producing maximum relaxant effect, showed that both of these natural compounds exert their relaxant effect by inhibiting the generation of reactive oxygen species (ROS) from endothelial and smooth muscle cells, blocking voltage-gated calcium channels, and increasing the release of nitric oxide (NO). The mediation of hypercontraction by nickel is due to the increased ROS and the influx of calcium through voltage-dependent calcium channels. These natural compounds are shown to counter the nickel-induced effects, appearing as effective ameliorators.

Conclusion: In this study, we found that quercetin and resveratrol act as ameliorators of nickel-mediated hypercontraction by decreasing ROS and enhancing NO release from endothelial cells.

Key words: amelioration, quercetin, resveratrol, hypercontraction, nickel

Corresponding author: Seemi Farhat Basir, Department of Biosciences, Faculty of Natural Science, Jamia Millia Islamia, New Delhi 110025, India
E-mail: sbasir@jmi.ac.in Phone: +91-9810597159

Introduction

Quercetin is a bio-flavonoid present mostly in the form of glycosides in more than 20 plant species (1). It is known for its antihypertensive and vasodilator effects; its relaxant effect has been shown on rat isolated aorta, rat portal veins, rat trachea and guinea pig isolated smooth muscles (2). Quercetin shows both endothelium-independent (3–5) as well as endothelium-dependent vasodilatory effects (5–7). It has also been shown that vaso-relaxation caused by quercetin in rat aortic rings is due to increased cGMP levels (6, 8).

Resveratrol, trans-3,5,4'-trihydroxy stilbenes, is a phytoalexin present in numerous types of nutrients that we consume daily. Resveratrol is isolated from dried roots of the Japanese and Chinese traditional medicinal plant *Polygonum cuspidatum* and used for treating many diseases, including skin inflammation, cardiovascular and liver diseases and fungal infections (9, 10). It has two isoforms (cis- and trans-resveratrol), with trans-resveratrol being the biologically active isoform. (11). Resveratrol is reported to cause relaxation of isolated human internal mammary artery, mesenteric artery and rat aorta (12, 13). Studies have shown that tissue systems, such as mesenteric and uterine arteries of guinea pig and porcine coronary vessel, retinal artery and abdominal aorta, were relaxed by treatment with resveratrol (14–16).

Nickel is reported to cause cardiovascular complications in various human and animal studies (17, 18). At low concentrations, nickel causes hypercontraction of dog heart and canine coronary arteries (19). This vasocontraction of canine coronary isolated arteries occurs through a tonic calcium activation mechanism (20). Substantial amounts of nickel are found in the urine of welders (21) as well as in their serum (22). Nickel exposure is reported to cause cardiac dysfunction, and superoxides generated by exposure of nickel are responsible for the pathogenesis of cardiac damage and vascular endothelial dysfunction (18, 23). Nickel sub-sulfide (Ni_3S_2) is reported to cause patchy denudation of arterial endothelial cells. It is also reported that nickel particles in Ni_3S_2-treated rats cause monoclonal proliferation of arterial smooth muscle cells (24).

Previously, we reported that nickel induces hypercontraction of isolated aortic rings at micro-molar concentrations, and the mechanism of action follows pathways that cause the generation of reactive oxygen species (ROS) by activating NADPH oxidase and the influx of calcium through voltage-gated channels (23). However, no study has been conducted to access the ameliorative potential of quercetin and resveratrol in isolated aortic rings hypercontracted due to exposure of nickel.

The present study therefore assessed the ameliorative effects of quercetin and resveratrol on isolated Wistar rat aortic rings that had been hypercontracted by incubation with nickel. We also explored the underlying mechanism of action by targeting pathways involved in nickel-induced hypercontraction.

Materials and Methods

Animals

The use of Male Wistar rats (n=30) weighing 300–400 g and around 3 months old was approved by the Institutional Animal Ethical Committee (no. 001/2016), Jamia Millia Islamia, New Delhi, India. Rats kept under constant temperature (27 ± 2 °C) with a standard light/dark cycle (12 h/12 h) were fed standard rat feed and provided drinking water *ad libitum*. Care of all animals was performed in compliance with the Guide for Care and Use of Laboratory Animals, published by the ILAR, National Research Council of the National Academies (N.W. Washington, DC, USA).

Solutions and drugs

Phenylephrine (PE), acetylcholine (ACh), verapamil, apocynin, quercetin, N-G-nitro-L-arginine methyl ester (L-NAME), dihydroethidium (DHE) and indomethacin, non-ionic polyoxyethylene surfactant (NP 40), sodium dodecyl sulfate (SDS) were procured from Sigma Chemicals (St. Louis, MO, USA). Resveratrol, sodium chloride, dextrose, magnesium sulphate, potassium dihydrogen phosphate, potassium chloride, calcium chloride, sodium bicarbonate and nickel chloride obtained from Merck (Maharastra, Mumbia, India) were used for preparation of Krebs buffer with the following composition (in mM): 120 NaCl; 25 $NaHCO_3$; 1.2 KH2PO_4;1.2 $MgSO_4$; 4.72 KCl; 11 $C_6H_{12}O_6$ and 2.5 $CaCl_2$. Phosphate-buffered saline (PBS) and 5% RIPA buffer (25 mM Tris HCl, 15 mM NaCl, 1% Na Dexylate, 1% NP 40, 0.1% SDS) were obtained from Merck India. Quercetin, indomethacin, resveratrol, apocynin and dihydroethidium were first dissolved in 10% Dimethyl Sulfoxide (DMSO) and diluted. The final vehicle concentration to which tissue was exposed was always less than 0.1%. At this concentration, DMSO did not interfere with PE.

Measurement of the aortic contractile activity

Wistar rats were anesthetized with pentobarbital (30 mg/kg bodyweight) (25). The thoracic aorta of the rat was removed and dipped in cold Krebs buffer. White fat covering the aorta was removed manually, and each aorta was cut transversally into 4- to 5-mm circular rings, taking care to avoid damaging the intact endothelium. Rings were mounted between 2 stainless steel wires in organ baths containing 15 ml Krebs medium, continuously bubbled with 95% O_2 and 5% CO_2 at 37 °C. All experiments were performed after an equilibration period of 60 min, with bathing medium renewed after every 15 min, which ruled out trauma or any other extraneous affects. Endothelium-intact aortic rings were stretched with a passive tension of 2.0 g. To select the resting tension, aortic rings were first stretched to 0.5 g, 1 g, 1.5 g and 2 g in different channels and then allowed to settle; 1 μM PE was then added to each channel after each stretching, and the response was noted. We observed a maximum response at 2 g of tension, so this was set as the resting tension for further experiments.

The tension was recorded using an isometric force transducer (MLT0420; AD Instruments, New South Wales, Australia) connected to a PC-based Data acquisition system from AD Instruments (PL3508 Power-Lab 8/35). Control contractions were induced with 1 μM PE to achieve a maximum response at this concentration (26). Each aortic preparation was challenged at the beginning of the experiment with 1 μM of ACh, and if the vasorelaxant response to ACh was greater than 50% of the PE-induced contraction, the aortic segment was considered to possess an intact endothelium.

Experimental design

Different concentrations of quercetin and resveratrol were added to Krebs buffer to study their relaxant effects on precontracted aortic rings. Aortic rings pre-challenged with 1 μM PE were incubated with quercetin and resveratrol for 40 min each. After incubation, 1 μM PE was added again, and the response of the aortic rings was recorded.

In the experiments involving the delineation of relaxant pathways of quercetin and resveratrol, the endothelium-intact aortic rings were first pre-incubated with either 1 μM verapamil (voltage-gated calcium channel blocker), 100 μM apocynin (NADPH oxidase inhibitor), 100 μM indomethacin (non-selective COX inhibitor) or 100 μM L-NAME (nitric oxide synthase [NOS] inhibitor) separately for the 40 min at their concentrations producing saturating effects, and the contraction was recorded over PE. In another set of experiments the endothelium-intact aortic were first pre-incubated with either 1 μM verapamil, 100 μM apocynin, 100 μM indomethacin or 100 μM L-NAME separately for 40 min, followed by incubation with quercetin and resveratrol

for another 40 min before the PE response was examined.

To study the ameliorative potential of quercetin and resveratrol, aortic rings were incubated first with 100 nM nickel for 40 min, followed by exposure to quercetin and resveratrol for another 40 min, before the PE response was examined.

In another series of experiments, the prophylactic potential of quercetin and resveratrol was studied. In these experiments, aortic rings were incubated with quercetin and resveratrol for 40 min, followed by exposure to nickel for another 40 min. The PE response was then recorded.

Estimation of total nitrite and calcium levels

Since NO depletion and increased calcium is involved in nickel-induced hypercontraction, we estimated the total nitrite and total calcium levels. Experiments with quercetin and resveratrol were performed under the same conditions (time period and exposure concentration) as for isometric tension studies in the presence of nickel using an organ bath system.

The effects of quercetin and resveratrol on total nitrite and calcium levels were studied following the methods reported earlier (27). Endothelium-intact aortic rings of approximately 8 mg (wet weight) were equilibrated with Krebs buffer for 60 min at 37 °C with continuous carbogen bubbling and exposed to 1 μM PE. Rings showing healthy contraction were washed and exposed to 30 μM resveratrol or 100 μM quercetin for 40 min. Aortic rings to which PBS had been added were used as vehicle controls. In another experiment, aortic rings were incubated with nickel for 40 min, followed by incubation with resveratrol or quercetin for another 40 min. After treatment, the tissues were frozen in liquid nitrogen immediately, crushed to a fine powder in with pre-chilled mortar and pestle and made into pellets. The tissue pellets were then suspended in 5% RIPA buffer (25 mM Tris HCl, 15 mM NaCl, 1% Na Dexylate, 1% NP 40, 0.1% SDS), manually chopped into fine pieces, homogenized and centrifuged at 1,500 *g* for 10 min at 4 °C. The supernatant obtained was stored at −80 °C and used for detection of total nitrite, using a Griess reagent kit (Merck, St. Louis, MO, USA; Cat no. G-4410-10G) and total calcium using a calcium detection kit (Cayman Chemical, Ann Arbor, MI, USA; Cat no. 700550) following the manufacturer's instructions.

Detection of ROS

This experiment was performed to detect the generation of ROS in aortic rings exposed to nickel and natural compounds (quercetin and resveratrol). Since ROS generation was found to be involved in nickel-mediated hypercontraction, and the natural compounds quercetin as well as resveratrol act as inhibitors of NADPH oxidase, the detection of ROS was performed. In this experiment, aortic rings with an intact endothelium were incubated with natural compounds (quercetin or resveratrol) alone or together with nickel for 60 min. Unexposed and nickel-exposed aortic rings were washed with Krebs buffer and fixed with 4% paraformaldehyde. Aortic rings were then cut into 5-μm-thick sections with a cryomicrotome. The aortic rings were incubated in 5 μM DHE for 30 min at 37 °C in the dark. These slides were kept on ice in a humidified chamber at room temperature throughout the entire experiment. Pictures were taken with a fluorescence microscope (DMLB, Nikon, Melville, NY, USA) and the I Vision software program (Nikon) at 20× and 40× magnification using a red filter.

Statistical analyses

All values are expressed as the mean ± the standard error of the mean (S.E.M.). Results were statistically analyzed using a paired *t* test or two-way repeated measures analysis of variance (ANOVA) followed by Duncan's multiple range test and Tukey's post-hoc test. Values of $P<0.05$ were considered statistically significant.

Results

Relaxant effect of quercetin and resveratrol on aorta

To assess the relaxant effect of quercetin, isolated aortic rings with intact endothelium were incubated with varying concentrations of quercetin (1 μM, 100 μM and 250 μM) for 40 min and contracted with 1 μM PE. Quercetin at 1 μM, 100 μM and 250 μM caused 20%, 29% and 29% inhibition of PE-induced contraction, respectively, compared to control (Fig. 1).

To assess the relaxant effect of resveratrol, endothelium-intact aortic rings were incubated for 40 min with varying concentrations of resveratrol (1 μM, 30 μM, 60 μM, 100 μM). Resveratrol caused 17%, 55%, 22% and 21% inhibition of PE-induced contraction, respectively, compared to control (Fig. 2).

The highest decrease in percentage contraction was noted at 100 μM by quercetin and 30 μM by resveratrol; these concentrations were therefore selected for delineation of the relaxation pathways.

Induction of relaxation by modulating resveratrol and quercetin with apocynin, verapamil, indomethacin and L-NAME in aortic rings

To delineate the mechanism of action of quercetin, isolated aortic rings were incubated with concentrations of various modulators producing saturating effects, such as verapamil, apocynin, indomethacin and L-NAME, followed by incubation with quercetin. Figure 1 shows the effect of quercetin (100 μM) on PE-induced aortic contraction in the presence of modulators. Aortic rings were incubated with modulators for 40 min followed by incubation with quercetin for 40 min, and then the PE-induced contraction was measured. Verapamil, apocynin and indomethacin caused a decrease in contraction, while L-NAME caused an increase in contraction when aortic rings were incubated with these agents for 40 min (Fig. 3).

Figure 3 shows the effect of quercetin (100 μM) on PE-induced contraction in the presence of modulators. The aortic rings incubated with 100 μM quercetin showed a 29% decrease in PE-induced contraction with respect to the control. Compared to quercetin alone, aortic rings incubated with 1 μM verapamil for 40 min

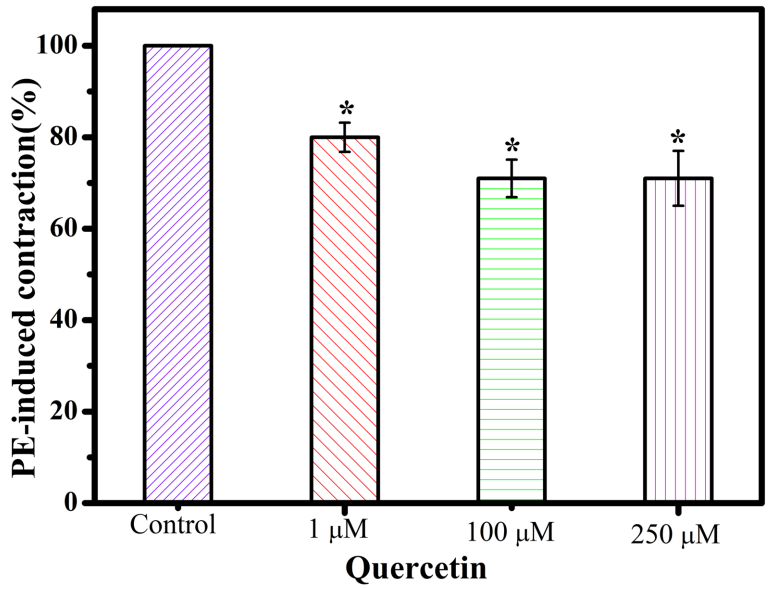

Fig. 1. Effect of varying concentrations of quercetin on isolated aortic rings over PE-induced contraction. *$P \leq 0.05$, a one-way ANOVA followed by Duncan's multiple range test. Results are presented as the mean ± S.E.M.; n=12 (n=number of rings).

followed by incubation with 100 µM quercetin for another 40 min did not show any significant change in the magnitude of contraction. In another set of experiments, aortic rings were exposed to 100 µM apocynin for 40 min, followed by incubation with quercetin for another 40 min. We observed a non-significant decrease (4%) in PE-induced contraction with respect to the quercetin-incubated aortic rings. Incubation of aortic rings

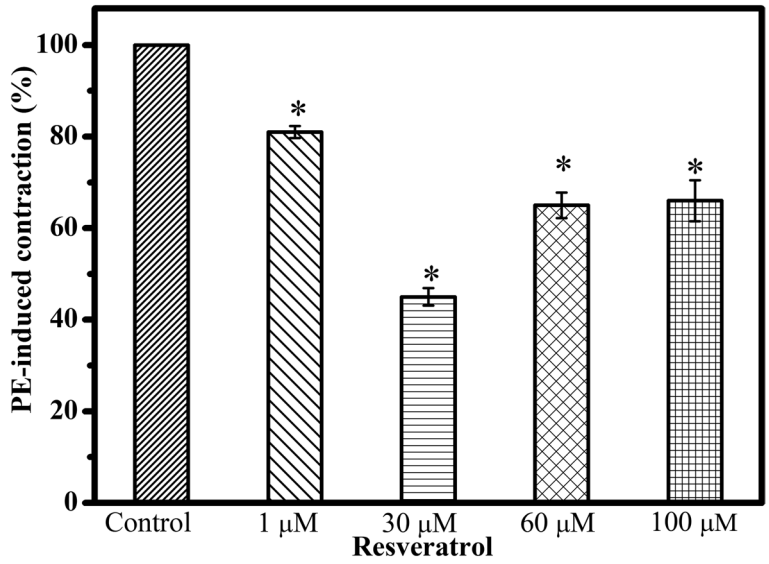

Fig. 2. Effect of varying concentrations of resveratrol on isolated aortic rings over PE-induced contraction. *$P \leq 0.05$, a one-way ANOVA followed by Duncan's multiple range test. Results are presented as the mean ± S.E.M.; n=15 (n=number of rings).

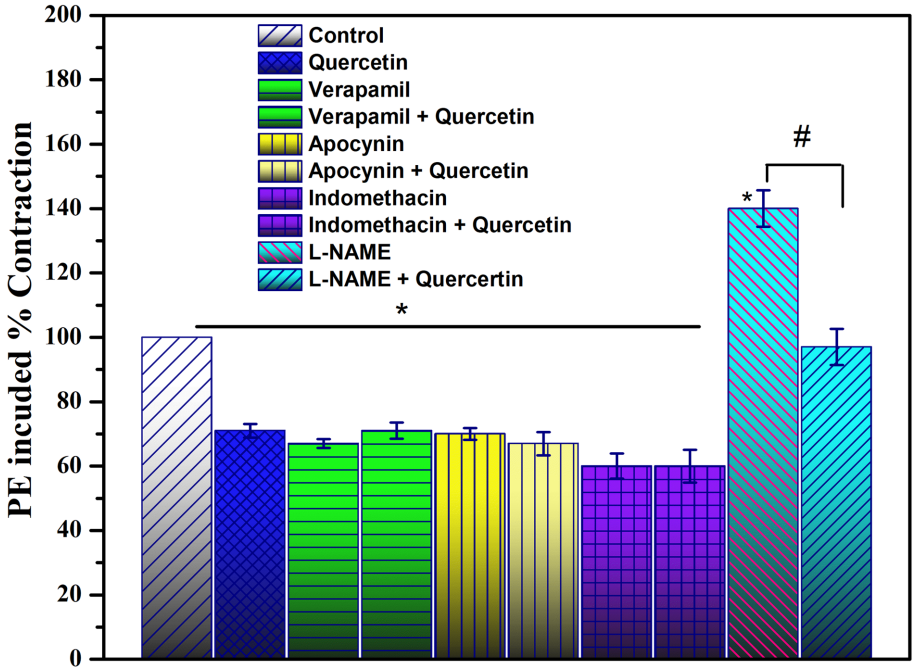

Fig 3. Effect of quercetin (100 µM) on PE-induced aortic contraction in the absence and presence of verapamil, apocynin, indomethacin and L-NAME. Quercetin, quercetin + modulators vs. control. *$P \leq 0.05$, an ANOVA followed by Tukey's post-hoc test. #$P \leq 0.05$ significance. Quercetin vs. quercetin + modulators, unpaired Student's *t*-test, results are presented as the mean ± S.E.M.; n=18 (n=number of rings).

with both indomethacin and quercetin caused a significant decrease in contraction (11%) with respect to incubation with quercetin alone. Our findings showed that an equal magnitude of relaxation is induced by quercetin in the presence or absence of verapamil and apocynin. However, the decrease in the percentage of PE-induced contraction by quercetin was greater in the presence of indomethacin than in its absence, suggesting different sites of action for quercetin and indomethacin. These results indicate that quercetin-induced relaxation is mediated by the same pathways as apocynin and verapamil but not by the same pathway as indomethacin.

The incubation of aortic rings with L-NAME (NO pathway blocker) and quercetin resulted in a 26% increase in contraction compared to aortic rings exposed to quercetin alone. This significant increase in PE-induced contraction in the presence of L-NAME indicates that quercetin-produced relaxation involves NO.

Aortic rings with intact-endothelium incubated with resveratrol (30 μM) for 40 min inhibited PE-induced contraction by 55% compared with control, as shown in Fig. 2. Figure 4 shows the effect of resveratrol (30 μM) on PE-induced aortic contraction in the presence of contractile pathway modulators, such as verapamil, apocynin, indomethacin and L-NAME. Compared to resveratrol alone, incubation of aortic rings with 1 μM verapamil followed by incubation with resveratrol 30 μM showed an insignificant change in contraction of 2%. Similarly, aortic rings incubated with 100 μM apocynin for 40 min followed by incubation with 30 μM resveratrol for 40 min also showed insignificant changes in contraction compared to resveratrol alone. Incubation of aortic rings with 100 μM indomethacin for 40 min followed by incubation with resveratrol for another 40 min similarly did not cause any significant change in contractile magnitude compared to resveratrol alone. These results indicate that resveratrol relaxes hypercontracted aorta by blocking voltage-gated calcium channels and by blocking the release of ROS from NADPH oxidase and COX.

However, the 21% increase in contraction observed in aortic rings incubated with L-NAME and resveratrol suggests the involvement of an NO-dependent component in relaxation caused by resveratrol.

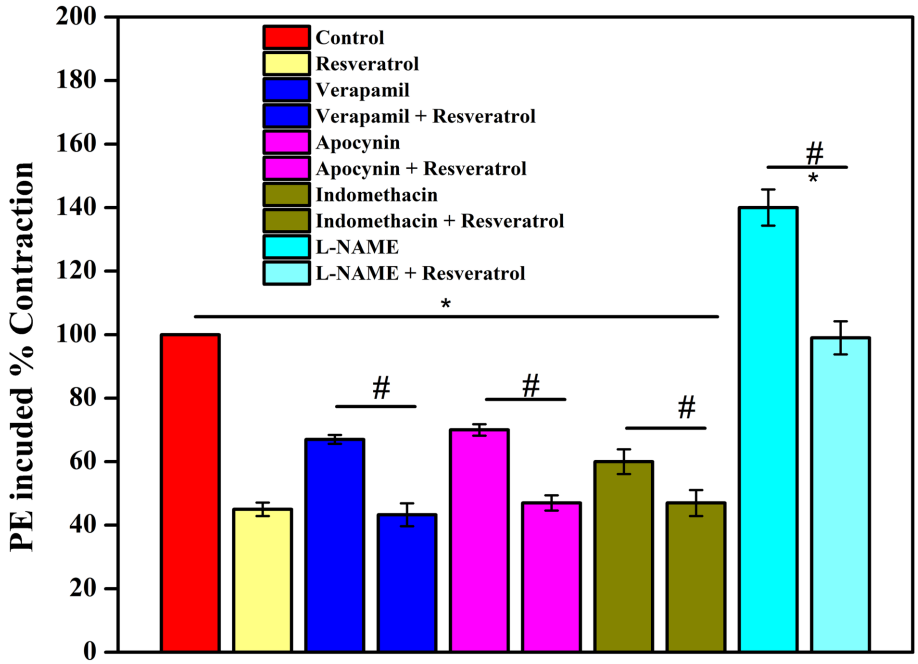

Fig. 4. Effect of resveratrol (30 μM) on PE-induced aortic contraction in the absence and presence of verapamil, apocynin, indomethacin and L-NAME. Resveratrol vs. control. *$P \leq 0.05$, an ANOVA followed by Tukey's post-hoc test. #$P \leq 0.05$ significance. Resveratrol vs. Resveratrol + modulators, unpaired Student's *t*-test, results are presented as the mean ± S.E.M.; n=18 (number of rings).

Effect of quercetin and resveratrol on unexposed and nickel-exposed aortic rings

To study the ameliorative effect of quercetin and resveratrol on nickel-exposed aortic rings, rings were incubated with 100 nM nickel for 40 min followed by exposure to quercetin and resveratrol. Aortic rings were then incubated with PE, and the contraction was recorded.

Figure 5 shows the magnitude of contraction elicited by quercetin and resveratrol for unexposed and nickel-exposed aorta. Aortic rings incubated with nickel (100 nM) for 40 min showed an 80% increase in contraction compared with control. We previously reported the complete mechanism of nickel-mediated contraction in aortic rings (24). In the current study, aortic rings incubated with nickel for 40 min followed by incubation with quercetin showed a 50% decrease in contraction with respect to the nickel-exposed aortic rings. Similarly, when aortic rings were incubated with nickel for 40 min followed by incubation with 30 μM resveratrol, we observed a 38% decrease in contraction compared to aortic rings incubated with nickel alone (Fig. 5).

Effect of incubation with resveratrol and quercetin on total nitrite/total calcium in nickel-exposed aortic rings

When nickel-exposed aortic rings that had been pre-incubated with PE were incubated with quercetin and resveratrol, the total nitrite concentrations were lower in the nickel-exposed aortic rings than in the control rings exposed neither to nickel nor natural compounds. However, under the same experimental conditions, aortic rings that were exposed to quercetin or resveratrol alone showed increased levels of nitrite (Fig. 6). Total nitrite concentrations in aortic rings co-incubated with resveratrol, quercetin and nickel remained unchanged compared with control aortic rings and higher than those in aortic rings exposed to nickel alone.

Aortic rings that were incubated with nickel showed a significant increase in total calcium levels compared with control. Total calcium levels in aortic rings incubated with nickel showed a 43% increase compared with control (Fig. 7). However, when aortic rings were incubated with nickel followed by incubation with quercetin or resveratrol, we observed a significant decrease in total calcium levels compared to aortic rings exposed to nickel alone. This suggests that quercetin and resveratrol may be responsible for blocking the calcium uptake through VGCC in aortic smooth muscle cells.

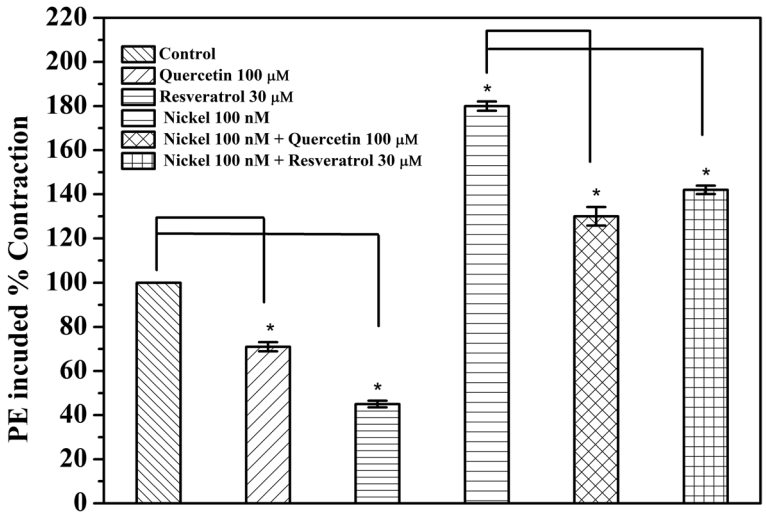

Fig. 5. Effect of quercetin and resveratrol on PE-induced contraction in unexposed and nickel-exposed aortic rings. Contraction of nickel-exposed aorta in the presence of natural compounds was compared with hypercontraction values. *$P \leq 0.05$, using Student's *t*-test and a one-way ANOVA along with Tukey's post-hoc test. Results are represented as the mean ± S.E.M. n=18 (number of rings).

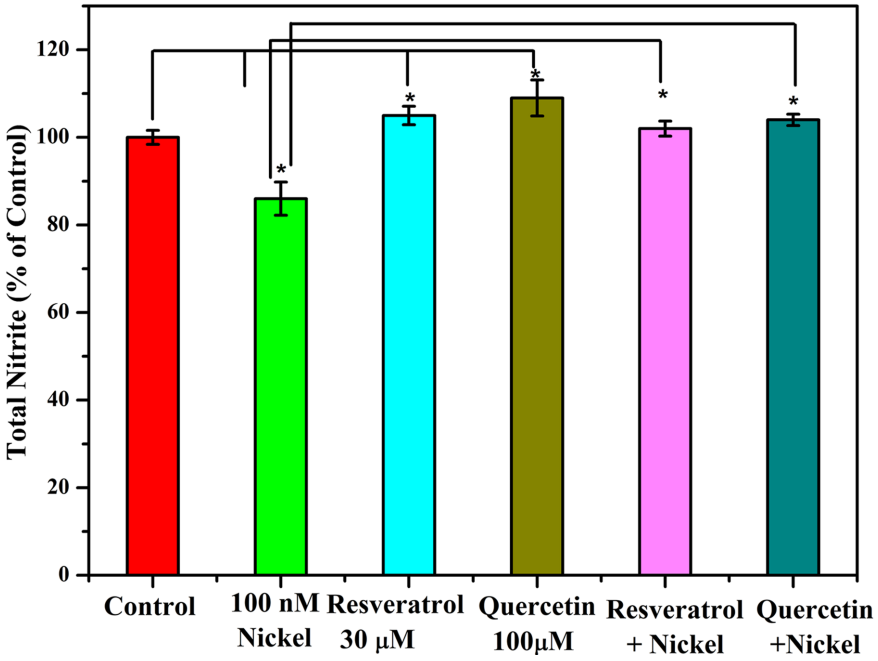

Fig. 6. Changes in total nitrite levels in nickel-exposed aortic rings. Effect of resveratrol and quercetin incubation on the total nitrite level in nickel-exposed aortic rings and unexposed aortic rings compared with control. Control aortic rings were exposed to neither nickel nor natural compounds. Results are expressed as the percent total nitrite levels compared to control. *$P \leq 0.05$, a one-way ANOVA along with Tukey's post-hoc test. n=18 (number of rings).

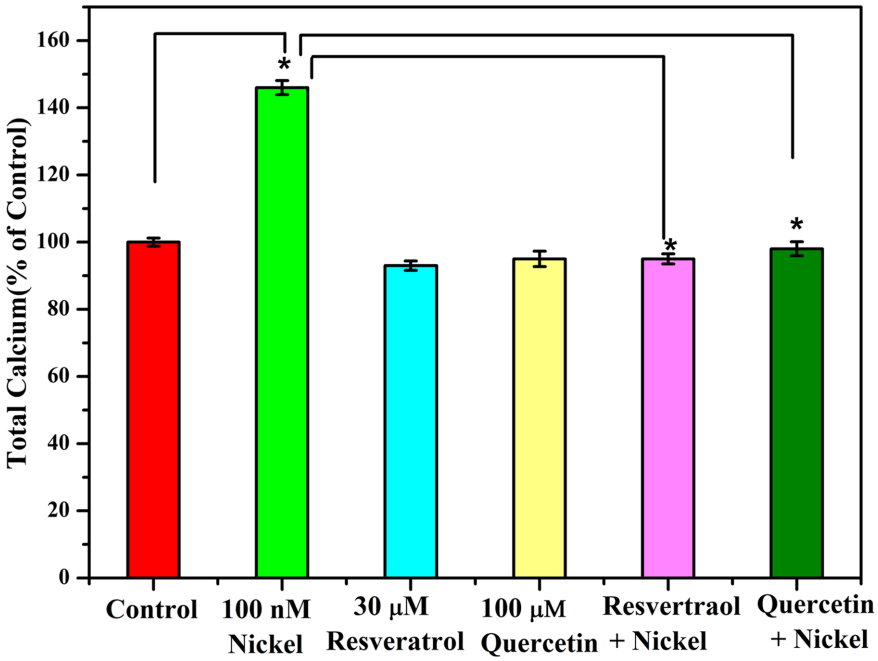

Fig. 7. Changes in total calcium level in nickel-exposed aortic rings. Effect of resveratrol, quercetin incubation on the total calcium level in nickel-exposed aortic rings and unexposed aortic rings compared with control. Control aortic rings were exposed to neither nickel nor natural compounds. Results are expressed as percent calcium levels as compared to control. Significant change vs. control. *$P \leq 0.05$, a one-way ANOVA along with Tukey's post-hoc test. n=18 (number of rings).

Effect of nickel on ROS generation

The generation of ROS due to nickel exposure was evaluated by dihydroethidium staining. Aortic rings exposed to nickel showed increased DHE staining, which indicates the generation of ROS, compared to control aorta. Figure 8 shows an ROS-scavenging effect in aortic rings co-incubated with quercetin, resveratrol and nickel. A comparison was made between images of aorta rings incubated with nickel and natural compounds (quercetin and resveratrol) and those of aortic rings incubated with natural compounds alone.

Discussion

Nickel at 100 nM is known to cause hypercontraction of uterine strips and cardiac muscles (19, 28). The reason for the rise in contraction is reportedly due to the uptake of extracellular calcium through voltage-dependent calcium channels. In our previous study, we reported that nickel at 100 nM caused an 80% increase in contraction of endothelium intact aortic rings compared with control. We found that the presence of nickel in aortic rings causes the influx of calcium through T-type calcium channels and increases the generation of ROS from endothelial cells as well as from smooth muscle cells (24).

The present study was planned to investigate the modulatory potential of plant-derived natural compounds in nickel-exposed rat aortic rings with intact endothelium. Plant-derived natural compounds reportedly induce the relaxation of smooth muscles by enhancing NO release, preventing endothelial dysfunction and acting as antagonists of the calcium channel pathways or ROS-generating pathways (29). We investigated the relaxant effect of quercetin and resveratrol on nickel-exposed and unexposed aortic rings and found that the aortic rings exposed to varying concentrations of quercetin showed significant inhibition of contraction compared with control. Quercetin at 1, 100 and 250 µM caused 20%, 29% and 29% inhibition of PE-induced

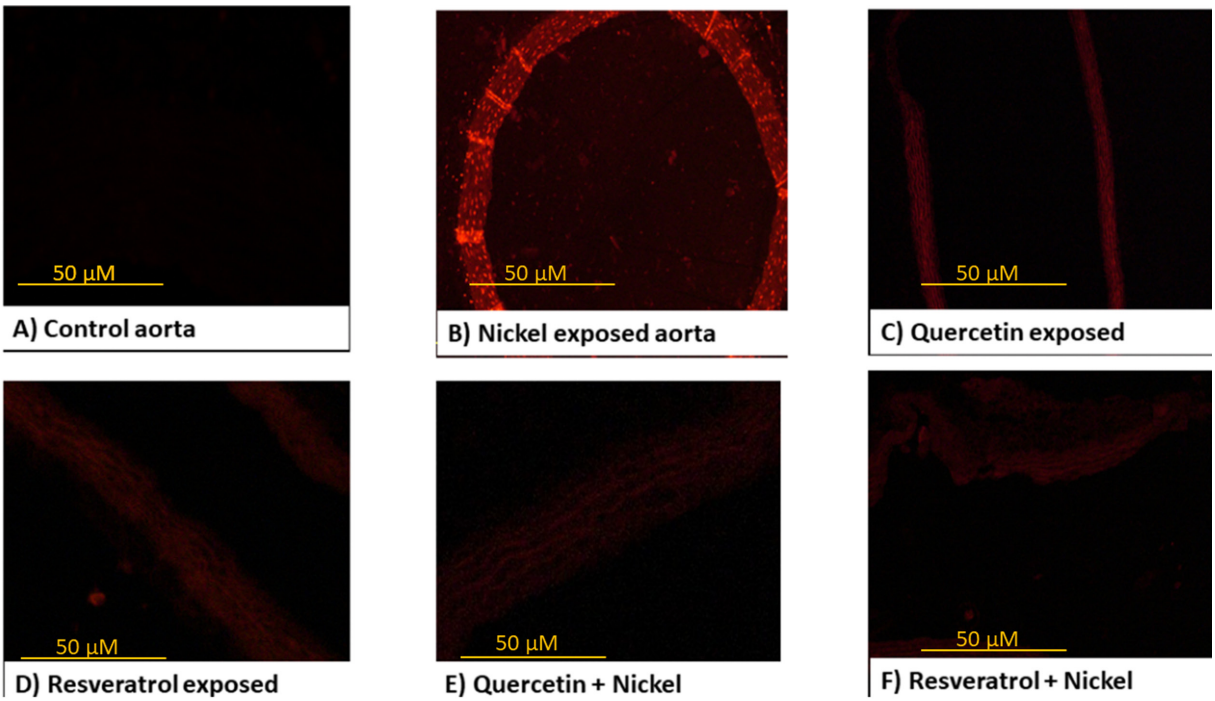

Fig. 8. ROS levels in aortic rings from (A) control aorta, (B) nickel-exposed aorta, (C) quercetin exposed aorta, (D) resveratrol exposed aorta, (E) quercetin + nickel-exposed aorta and (F) resveratrol + nickel by dihydroethidium (DHE) fluorescence (×10).

contraction respectively, compared to control. Endothelium-intact aortic rings were also exposed to varying concentrations (1, 30, 60 and100 μM) of resveratrol, which caused a 19%, 55%, 22% and 21% decrease in contraction compared with control (Figs. 1 and 2). Our results indicate that resveratrol at low concentrations caused maximum relaxation. Several studies have shown resveratrol to have a biphasic function, depending on the dose. At lower concentrations, it functions as an antioxidant, whereas at higher doses, it functions as a pro-oxidant (30, 31).

To gain insight into the mechanism of action underlying the relaxation, quercetin and resveratrol were administered along with various modulators at concentrations that showed saturating effects. Quercetin demonstrated a biphasic response in isolated aorta that caused a brief increase in basal tone followed by a sustained, long-lasting relaxant effect (3). This transient increase in basal tone by quercetin is reportedly due to the activation of L-type channels, which leads to the enhanced influx of calcium (32). The sustained relaxation response caused by quercetin is attributed to the release of NO from endothelial cells (8).

L-NAME is known to inhibit NOS and therefore block release of NO, a potent smooth muscle relaxant (33). When we incubated aortic rings with L-NAME followed by quercetin, we observed a 27% increase in the PE-induced contraction over that shown by quercetin alone. This suggests that the relaxation effect of quercetin seen in an earlier experiment (Fig. 3) is attenuated by the presence of L-NAME (Fig. 3). The increase in vasocontraction is due to the production of endothelium-derived contracting factors and the decrease in NO in the presence of L-NAME (34). Similarly, our results also suggest that quercetin is responsible for enhancing the NO release, and indeed, quercetin was previously reported to cause restoration of NO (35–37).

Quercetin is reported to exert an antagonistic effect on voltage-gated calcium channels (38). It has been stated that the relaxant effects of quercetin are due to blockage of voltage-gated calcium channels and by increasing the release of NO through endothelial NOS (14, 39). We too observed that quercetin causes blockage of calcium channels, as incubation of aortic rings with verapamil (a calcium channel blocker) followed by exposure to quercetin led to no significant change in PE-induced contraction compared with quercetin-exposed aorta. This means that quercetin and verapamil both act on the same pathway in the contractile machinery.

Apocynin (NADPH oxidase inhibitor) is reported to cause direct scavenging of ROS in vascular smooth muscle cells and inhibits the release of superoxide anion by NADPH oxidase (40, 41). In the present study, aortic rings incubated with 100 μM apocynin for 40 min followed by quercetin did not show any significant change in PE-induced contraction, suggesting that apocynin and quercetin act via the same pathway i.e. inhibition of ROS generation (Fig. 3). Quercetin may therefore be involved in the reversal of nickel-mediated contraction of aortic rings by enhancing the NOS activity or by enhancing NO and inhibiting ROS generation (42, 43). However, when aortic rings were incubated with indomethacin (a non-selective COX inhibitor) for 40 min followed by quercetin, we observed a significant decrease in PE-induced contraction compared with quercetin alone. This implies that indomethacin and quercetin have different sites of action, as was earlier suggested by Morales et al. (44, 45).

Resveratrol at 30 μM caused 55% relaxation in endothelium-intact aorta compared with control (Fig. 2). This concentration was further used to delineate the mechanism of action. A non-significant change in relaxation was seen in aortic rings incubated with apocynin (an inhibitor of NADPH oxidase) and resveratrol compared with resveratrol alone (Fig. 4), indicating that resveratrol mediates relaxation via the same route as apocynin, i.e. by inhibiting ROS production. A number of studies have reported that resveratrol causes a reduction in ROS at the cellular level (46). Resveratrol has been previously reported to cause downregulation of NADPH oxidase (47). However, Spanier et al. reported that resveratrol caused the inhibition of NADPH oxidase in cardiovascular tissues and linked this effect to its ROS-scavenging properties (48). We observed a

21% increase in contraction when aortic rings were incubated with L-NAME and resveratrol, indicating that an NO-dependent component is involved in relaxation induced by resveratrol. The mechanism underlying the endothelium-dependent relaxation induced by resveratrol has already been described by Chen and Pace-Asciak, who reported that the vasorelaxant effect of resveratrol is mediated by NO (14).

When aortic rings were incubated with verapamil (L-type calcium channel blocker) for 40 min followed by incubation with resveratrol, we observed a non-significant difference in PE-induced contraction compared with the incubation of aortic rings with verapamil and resveratrol (Fig. 4). As verapamil is a calcium channel blocker, its presence along with resveratrol does not markedly influence PE-induced contraction compared with resveratrol alone. This suggests that resveratrol may also be a calcium channel blocker. Resveratrol causes inhibition of calcium influx through L-type calcium channels and stops the release of calcium from endoplasmic reticulum (49). Aortic rings incubated with indomethacin (a non-selective COX inhibitor) followed by resveratrol showed no change in contractile magnitude over PE (Fig. 4) compared to resveratrol alone, suggesting that both indomethacin and resveratrol exert their inhibitory response by working on COX-mediated pathways. Resveratrol action therefore seems to be dependent on COX. In cultured cells, the anti-inflammatory effect of resveratrol has been attributed to inhibition of the COX-2 pathway (50, 51). It was also shown that resveratrol inhibits the expression of COX-2 in both *in vivo* and *in vitro* models (52, 53). Resveratrol thus appears to be a potential relaxant molecule that relaxes the aorta via multiple pathways, such as by quenching ROS generated by NADPH oxidase as well as by generating COX-mediated prostanoids, blocking the influx of calcium through voltage-gated calcium channels and enhancing the bioavailability of NO (Fig. 4) (54–56). Figure 9 is a diagrammatic representation showing that aortic rings exposed to nickel had an enhanced release of ROS and decreased release of NO along with the influx of calcium, thus contributing to hypercontraction of the aorta. However, natural compounds appear to mitigate the hypercontraction of the aorta, causing relaxation.

The present study evaluated the utility of quercetin and resveratrol as ameliorators of nickel-induced hypercontraction by exposing aortic rings to them and incubating them with nickel at its saturating concentration. Nickel at 100 nM induced 80% contraction in aortic rings compared with control; this enhancement of

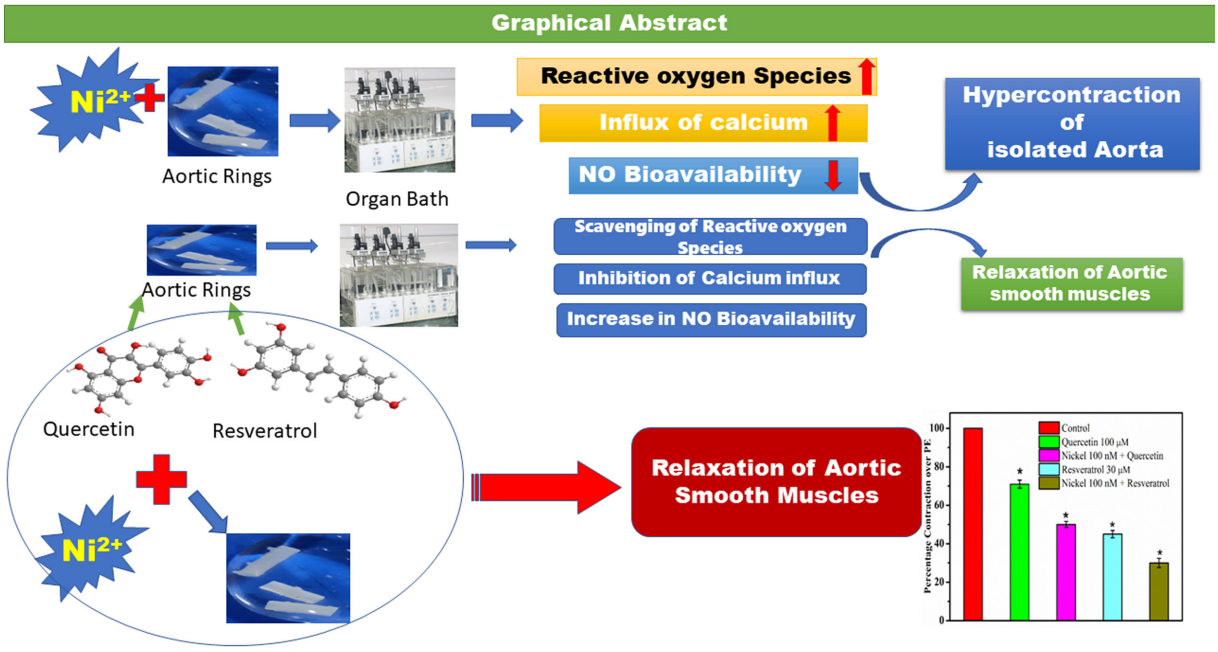

Fig. 9. Schematic figure depicting the study flow.

contraction was due to the increased influx of extracellular calcium through voltage-gated calcium channels and release of ROS (24). We further found that quercetin caused 50% inhibition of PE-induced contraction compared with aortic rings incubated with nickel alone, and 38% inhibition of PE-induced contraction was observed in aortic rings exposed to nickel followed by exposure to resveratrol compared with nickel alone (Fig. 5). This relaxation of nickel-exposed aorta was greater than the relaxation induced by quercetin and resveratrol in aorta not exposed to nickel, suggesting that both natural compounds may act as better ameliorators of nickel-induced hypercontraction in aortic smooth muscles. Other researchers have also studied the ameliorative potential of terpenes, such as carvone, eugenol and linalool, in arsenic- and mercury-exposed aortic tissues (57).

In our evaluation of the effect of nickel on NOS and the restorative effects of quercetin and resveratrol, aortic rings exposed to nickel showed a decrease in total nitrite levels compared with control. This is because nickel causes either a decrease in bioavailability of NO in aortic rings or endothelial dysfunction. We estimated the total nitrite level in aortic rings and found a higher nitrite level in aortic tissues exposed to quercetin and resveratrol than in nickel-exposed aortic rings, indicating that these natural compounds exert NO-enhancing activity of endothelial NOS compared with control (Fig. 6).

Nickel is reported to cause influx of calcium through voltage-gated calcium channels (20, 24). To assess this effect, the total calcium level in aortic rings exposed to nickel was measured. We found that the total calcium level in aortic rings incubated with nickel was 43% higher than in control (Fig. 7), while a decrease in the calcium level was observed in aortic rings that were co-incubated with nickel and natural compounds (quercetin or resveratrol). This indicates that quercetin and resveratrol act as blockers of calcium channels in smooth muscles, thus supporting our organ bath findings, wherein resveratrol and quercetin blocked voltage-gated calcium channels. We observed no evidence of fluorescence in aortic rings co-incubated with nickel along with quercetin and resveratrol compared with aortic rings incubated with nickel alone, signifying that these natural compounds act either as effective ROS scavengers or that they may cause blockage of NADPH oxidase (Fig. 8).

Conclusion

Quercetin and resveratrol proved to be good ameliorators of nickel-mediated contraction. Both natural compounds caused relaxation of aortic rings by decreasing ROS and enhancing NO release from endothelium cells. Some of the relaxation effects of quercetin and resveratrol are also mediated by their calcium channel-blocking properties. Thus, quercetin and resveratrol can be beneficial for reducing hypercontraction induced by nickel and possibly that induced by other metal pollutants as well.

Authors' Contribution

All authors were involved in the conception, design and interpretation of the work; the first author performed the experiments. All authors have read and approved the manuscript and declare that all data were generated in-house with no paper mill used.

Consent to Participate

Not applicable for this study, as we did not perform a study with human subjects.

Consent for Publish

Not applicable for this study, as we did not perform a study with human subjects.

Ethical Approval

Approval was obtained from the university ethics committee of Jamia Millia Islamia (New Delhi, India), no. 001/2016.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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