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Original Article

Effects of quercetin on exercise performance, physical activity and blood supply in a novel model of sustained hind-limb ischaemia

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Abstract

Background: There are currently few effective drugs to treat the leg symptoms of peripheral arterial disease (PAD). Previous studies have suggested that the nutraceutical, quercetin, can improve exercise performance and reduce pain sensitivity in healthy mice and improve blood supply in a rodent model of acute hind-limb ischaemia. These models may not be relevant to people with PAD. The aim of this study was to examine the effect of quercetin on exercise performance, physical activity and blood supply in a novel mouse model of sustained hind-limb ischaemia.

Methods: Hind-limb ischaemia was induced in 6-month-old male apolipoprotein E-deficient mice using a novel two-stage surgical procedure. Five days after induction of ischaemia, mice were allocated to commence dietary quercetin or a control diet for 4 weeks. The primary outcome was exercise performance evaluated using a treadmill test. Other outcomes included physical activity, estimated by an open field test, and hind-limb blood supply, assessed by laser Doppler monitoring.

Results: A sustained reduction in relative limb blood supply ($P < 0.001$) was achieved consistently in all 48 mice before allocation to a control ($n = 24$) or quercetin ($n = 24$) diet. Quercetin did not improve exercise performance ($P = 0.785$), physical activity ($P = 0.151$) or relative limb blood supply ($P = 0.954$) over the 4-week assessment period.

Conclusion: These data suggest that quercetin does not improve exercise performance, physical activity or limb blood supply in mice with sustained hind-limb ischaemia, and therefore is unlikely be an effective treatment for PAD.

Surgical relevance

This paper describes the testing of a nutriceutical in a novel patient-relevant model of limb ischaemia. The study suggests that quercetin did not improve limb ischemia or ambulation ability in this clinically relevant model.

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Introduction

Peripheral arterial disease (PAD) is a common cause of leg pain, walking impairment, low physical activity. and poor health-related quality of life[1–3]. Cilostazol is the only currently available drug therapy for the leg symptoms of PAD, but is not commonly used or currently recommended by European practice guidelines[4].

The flavonoids are a group of polyphenols found in some foods[5]. Previous reports have suggested that the flavonoid quercetin can improve recovery of limb blood flow and increase capillary density[6], reduce muscle atrophy[7,8], and limit oxidative stress and reduce proinflammatory cytokine concentrations in skeletal muscle[9,10] in animal models. Cytokines, including interleukin (IL) 6 and tumour necrosis factor (TNF) α, have been detected at high levels in the skeletal muscle of patients with PAD[11]. High concentrations of these cytokines and low availability of nitric oxide have been suggested to contribute to the most common and debilitating symptoms of PAD: poor ambulatory performance[12] and muscle pain[11]. Quercetin has been reported to improve exercise performance in healthy mice[13] and to reduce acid-induced pain stimulation[9]. This is potentially achieved through a reduction of TNF-α and increases in endothelial nitric oxide production[6]. These research findings suggest that quercetin may be an effective treatment for PAD; however, the relevance of these previous studies to people with PAD is unclear.

Previous animal studies on PAD have a number of limitations, including the use of an acute ischaemia model in which blood supply recovers rapidly, investigating young rodent strains with excellent angiogenesis ability, poor study design, and employing

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outcome measures unrepresentative of the clinical situation, as reviewed in detail previously[14]. A novel two-stage surgical hind-limb ischaemia (HLI) mouse model was developed previously to simulate human PAD better[15]. This two-stage model has sustained HLI without spontaneous recovery, and also illustrates impaired exercise performance[15]. The aim of this study was to test the effect of quercetin supplementation on exercise performance, physical activity and blood supply in the two-stage HLI rodent model.

Methods

Male apolipoprotein E-deficient (*ApoE*$^{-/-}$) mice aged 18–22 weeks were purchased from the Animal Resources Centre (Canning Vale, WA, Australia) and acclimatized for 4 weeks at the animal facility at James Cook University. Mice were housed, one per cage, in individually ventilated techniplast cages with corn cob bedding, on a 12/12-h light/dark cycle, at a relative humidity of 55 ± 2 per cent and a temperature of 23 ± 2°C. Before experiments, mice were maintained on normal laboratory chow and water *ad libitum*. All conditions were kept constant between experimental groups, with the exception of quercetin being incorporated into the diet of the intervention group. Ethics approval was obtained from the James Cook University Animal Ethics Committee (A2352). Mice were monitored twice-daily after surgery.

Study design and outcome assessment

Unilateral HLI was induced in 22–26-week-old mice through two sequential operations on days 1 and 14 after the start of the experiment, as described previously[15]. Mice were then allocated to two experimental groups with similar mean baseline exercise performance and similar levels of ischaemia, as indicated by laser Doppler blood flow measurements on postoperative day 14. Animals in group 1 received a diet that contained quercetin, and those in group 2 received an otherwise identical control diet that did not contain quercetin. Mice were allowed to recover from surgery, and 5 days later, on day 19, were fed either the diet containing quercetin or the control diet until the end of the experiment (day 51). Research was conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines[16].

Exercise performance was assessed in a treadmill test (TMT), performed at baseline (day 0) and on days 24, 34 and 44 after the start of the experiment. Physical activity was monitored through open field tests performed on day 44, and limb pressure sensitivity was assessed on day 48. Hind-limb blood flow was measured on days 0, 13, 15, 28, 35 and 49 (*Fig. S1*). Blood samples were taken from the tail vein on days 0, 30 and 51 (endpoint). Bodyweight was measured weekly. At the end of the experiments, mice were killed by carbon dioxide asphyxiation.

Induction of sustained hind-limb ischaemia

Unilateral HLI was created with a two-stage surgical procedure, as described in detail previously[15]. Briefly, in the first operation two hygroscopic ameroid constrictors with an internal diameter of 0.25 mm (Research Instruments SW, Escondido, CA, USA) were placed around the femoral artery, one inferior to the internal iliac artery branch and one superior to the deep femoral artery branch. Fourteen days later, the femoral artery was ligated using 6/0 silk sutures at two sites, one adjacent to each ameroid constrictor, and the artery was resected along with both ameroid constrictors and any adherent collateral branches. The incisions were closed and animals were monitored for recovery.

Experimental groups

Control mice received a diet containing 20 per cent protein, 4.8 per cent fat, 4.8 per cent crude fibre, 0.8 per cent calcium, 0.7 per cent phosphorous, 0.36 per cent salt, and 14-MJ/kg digestible energy (Specialty Feeds, Glen Forrest, WA, Australia). The intervention group received quercetin (Q4951; Sigma, Castle Hill, NSW, Australia), 0.16 per cent w/w, incorporated into a diet otherwise identical to the control diet. Based on an average food consumption of 3.45 g/day[17], mice in this study consumed approximately 185 mg/kg quercetin daily.

Assessment of exercise performance with a treadmill test

This was the primary outcome for the study. A TMT was performed using a six-lane treadmill (Excer-3/6; Columbus Instruments, Port Macquarie, NSW, Australia), as described previously[15]. The test measured distance travelled to fatigue, comparable with maximum walking distance commonly measured in clinical trials[15,18]. Mice were acclimatized to the treadmill over a 3-day period for 15 min each day before experiments took place. Before each TMT, mice were fasted for 2 h to avoid postprandial effects. Over the first minute, the speed was increased to 15 m/min and shock grids were turned on. Total distance travelled was recorded from the start of running at 10 m/min, and mice were considered fatigued once they had 10 separate visits to the shock grid.

Assessment of physical activity with an open field test

The open field test measured voluntary activity and has been compared to a 6-min walk test used in clinical trials, although it is more relatable to an objective measure of physical activity such as an accelerometer[19]. Mice were acclimatized in the building where open field experiments had occurred 3 days before recording. The animals were placed in an open field cube (40 × 40 × 40 cm, divided into an outer field and a central field of 20 × 20 cm), and their movement was recorded for 20 min with a Logitech quick capture webcam® (Logitech Australia Computer Peripherals Pty Ltd, Marleston, South Australia). Analyses of open field recordings were performed using TopScan Lite 2.0 software. Total distance travelled and total velocity were recorded for each mouse, to allow for comparison between groups.

Assessment of hind-limb pressure sensitivity

Limb pressure sensitivity was measured using a pressure application device (PAM) (Ugo Basile, Gemonio, Italy), as reported previously[15]. Limb pressure sensitivity is a model of induced pain response that has been used to assess pain due to ischaemia[20]. Mice were restrained in a supine position and pressure was applied to the hind limb with a force transducer at a rate of 20 g/s. The point at which mice withdrew the limb, vocalized, or had twitching of whiskers in response to the device was considered the endpoint. The peak force applied immediately before the endpoint was considered the limb withdrawal threshold. The data were normalized as the limb withdrawal threshold ratio of the left (ischaemic) and right (contralateral) hind limb, to allow comparison between groups.

Assessment of blood flow with laser Doppler perfusion imaging

Blood flow was measured in both hind limbs of mice using laser Doppler perfusion imaging (LDPI) (Moor Instruments, Axminster,

UK). Isoflurane (Henry Schein, Columbus, OH, USA) was administered at 3 per cent in oxygen at a flow rate of 1 l/min. Hind limbs were taped with the plantar surface facing downward, and Doppler imaging was performed. The colour flux image produced by LDPI was analysed to produce perfusion units using computer software (MoorLDI version 3.08, Moor Instruments, Axminster, UK). These perfusion units were then normalized as left to right hind-limb ratios to allow comparison between groups. The coefficient of variation of intraobserver and interobserver reproducibility of measurements of limb perfusion were 3.5 per cent ($n = 43$) and 5.1 per cent ($n = 43$) respectively.

Preparation of plasma samples

Blood samples were centrifuged at 4°C, 2000*g* for 10 min, and supernatant was transferred to microfuge tubes for further centrifugation at 15 000*g* for 10 min. Supernatant plasma was aliquoted into microfuge tubes, snap-frozen in liquid nitrogen, and stored in −80°C freezers.

Total plasma nitrate and nitrite concentration

Total nitrate and nitrite concentrations were quantified in the plasma as a measure of nitric oxide concentration. Analysis was performed on plasma samples collected from mice at baseline and day 30 after the start of the experiment using a nitric oxide fluorometric assay kit (ab65327; Abcam, Melbourne VIC, Australia) according to the instructions for use. Fluorescence was measured at an excitation and emission of 360 and 450 nm, and converted to millimolar concentrations using a standard curve.

Quantification of plasma cytokines

Plasma IL-6, IL-10, IL-12 and TNF-α concentrations were measured at baseline and endpoint using a cytokine magnetic bead panel (MILLIPLEX®, MCYTOMAG-70K®, Melbourne, Australia) according to instructions for use. Briefly, 25-µl plasma samples were incubated overnight to allow for binding of cytokines to bead antibodies. Samples were incubated with detection antibodies and streptavidin–phycoerythrin, and the plate was run on a MAGPIX® machine (Abacus dx, Meadowbrook, QLD, Australia). Concentrations were converted to picograms per millilitre using a standard curve.

Sample size calculation

The required sample size was calculated based on the effect size reported for quercetin improving exercise performance in a TMT in healthy mice[13]. In that study, 16 mice were given quercetin (25 mg/kg daily) for 7 days and achieved a mean(s.e.m.) of 37(9.5) per cent longer distance during the test than controls[13]. The sample size calculation for the present study was two-tailed; power was set to 0.80 and the α value was set at 0.05. Based on these values, the minimum sample size required was 20 mice per group.

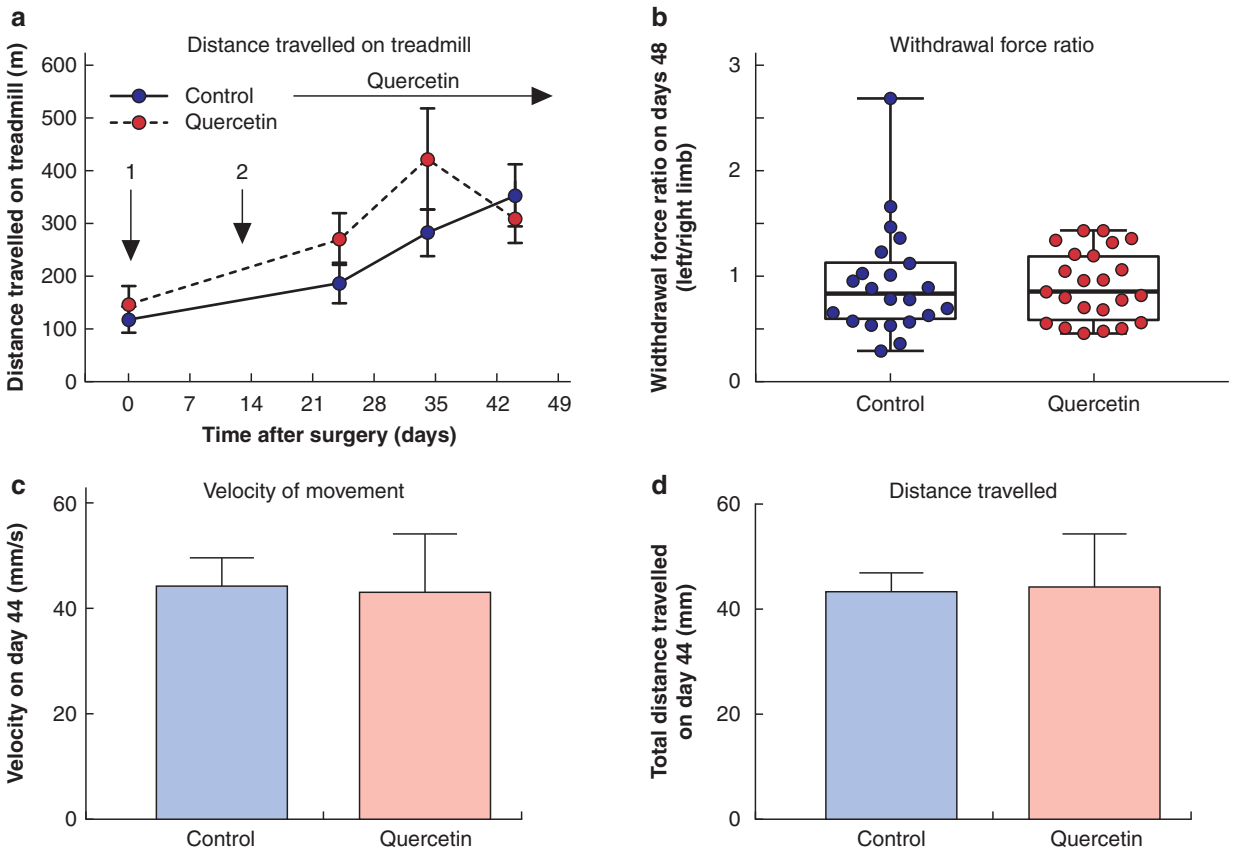

Fig. 1 Effect of quercetin on ambulatory performance, pressure sensitivity and voluntary activity

a Distance travelled in a treadmill test. 1, surgical procedure to implant ameroid constrictors; 2, surgical procedure to ligate femoral artery and establish hind-limb ischaemia. Data were analysed using a linear mixed-effects model, and were log-transformed during statistical analysis to conform to model assumptions, although raw values are shown here. Log-transformation of the data did not change statistical findings from the primary analysis. Values are mean(s.e.m.). **b** Force applied to hind-limb paws immediately before limb withdrawal or signs of distress, expressed as a ratio of left (ischaemic) to right (contralateral) limb. Median values, interquartile ranges and ranges are denoted by horizontal bars, boxes and error bars respectively. **c** The mean(s.e.m.) velocity at which mice moved during an open field test in the whole area, and specifically within the exterior and centre zones. **d** Mean(s.e.m.) distance travelled during an open field test in the whole area, and specifically within exterior and centre zones. There were 23 animals in the quercetin group and 22 control animals. For *P* values, see main text.

Sample size was increased by 20 per cent to account for potential dropouts due to surgical complications or mice welfare concerns.

Statistical analysis

Data were tested for gaussian distribution using the D'Agostino and Pearson test. Normally distributed data were expressed as mean(s.e.m.) values, and non-normally distributed data as median (i.q.r.) values. Differences between groups were assessed using unpaired two-tailed *t* tests or Mann–Whitney *U* tests, as appropriate. Plasma cytokine values that did not fall within the assay detection range were excluded. Blood flow, distance travelled and bodyweight were measured at multiple time points throughout the study, and compared between groups using linear mixed-effects models. Blood flow, distance travelled, or bodyweight and time were set as fixed effects, and variation between individual mice was set as a random effect. The interaction of time and treatment was considered in order to assess significance in these tests. Mice were allocated to control or quercetin-enriched diet 5 days after the second HLI operation; however, data for the individual groups of mice are presented and analysed from the start of the experiment (before HLI induction) to illustrate the overall effect of ischaemia on outcomes. Model fit was assessed through examination of the spread of standardized residuals and normal Q-Q diagnostic plots. Where diagnostic plots suggested that the assumptions of linear mixed effects were not met, data were log-transformed. Where potentially influential outliers were identified (defined as more than 3 standard deviations from the mean of all residuals for linear mixed-effects models), sensitivity analyses were conducted excluding these outliers. Differences were considered statistically significant when $P < 0.050$. Statistical analyses were performed using GraphPad Prism® version 6 (GraphPad Software, San Diego, CA, USA) and RStudio (https://rstudio.com).

Results

A total of 48 mice commenced the experiment, 24 mice per group. Three mice (one from the intervention group and two controls) did not wake up from anaesthesia following HLI induction, and were excluded from the study as the intervention had not commenced. Bodyweight of mice did not change significantly over the duration of the experiment and was similar in both groups (*Fig. S2*). The exclusion of outliers identified in bodyweight data did not affect the statistical findings.

The mean(s.e.m.) distance travelled during the TMT increased significantly over time in mice allocated to control (117.6(24.8), 187.1(39.0), 283.3(43.5), 353.8(59.3)) and quercetin (147.7(35.0), 270.0(50.1), 422.6(96.3), 310.3(46.9)) diets on days 0, 24, 34 and 44 respectively ($P < 0.001$) (*Fig. 1a*), but there was no difference between intervention and control animals ($P = 0.785$). There was

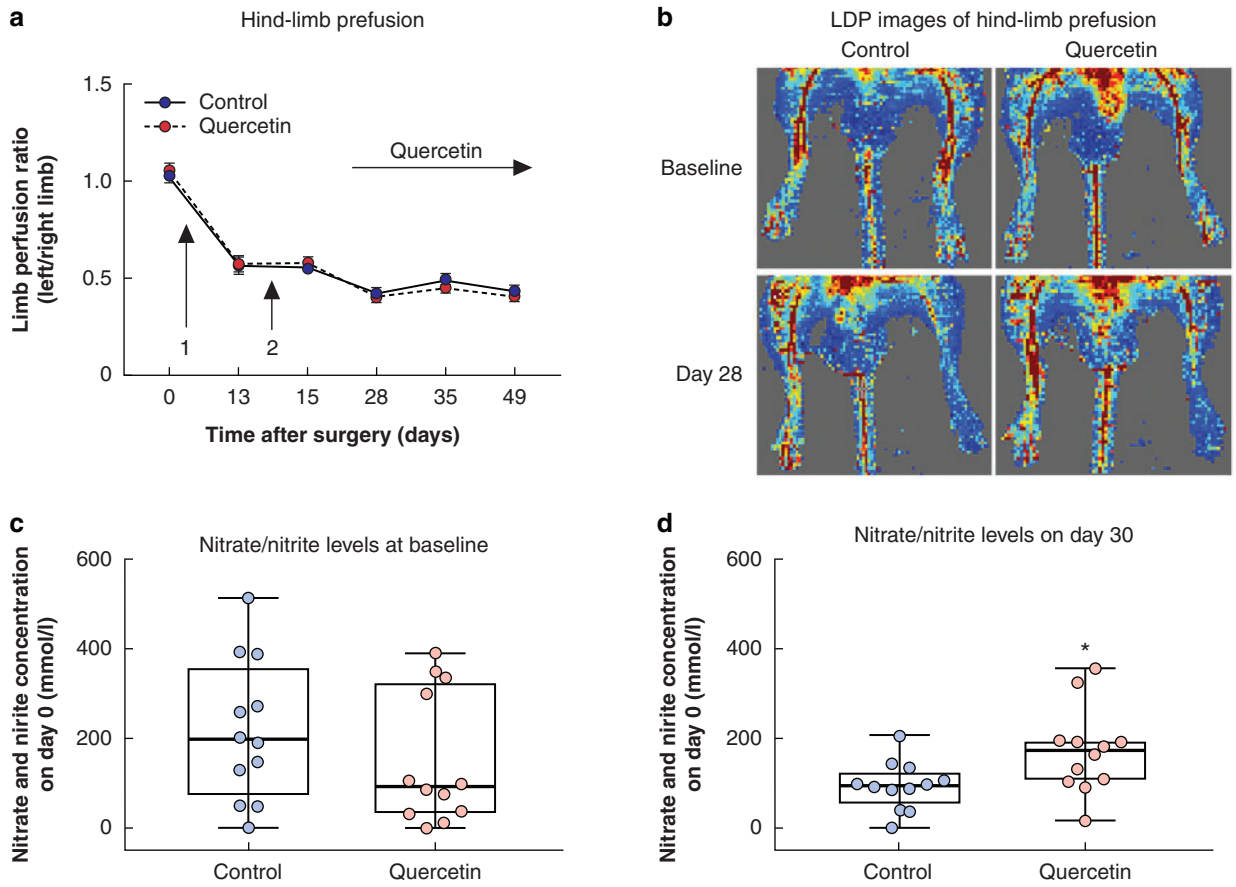

Fig. 2 Effect of quercetin on hind-limb perfusion measured by laser Doppler perfusion imaging and plasma nitrate and nitrite concentrations

a Hind-limb perfusion decreased after surgery in both groups, but did not differ between groups (quercetin, $n = 23$; controls, $n = 22$). 1, surgical procedure to implant ameroid constrictors; 2, surgical procedure to ligate femoral artery and establish hind-limb ischaemia. Data presented as mean(s.e.m.) values and analysed using a linear mixed-effects model. **b** Representative laser Doppler perfusion (LDP) images of hind-limb perfusion at baseline and day 28. **c** Median (i.q.r.) total plasma nitrate and nitrite concentrations did not differ between groups at baseline (quercetin, $n = 12$; controls, $n = 12$). **d** Median (i.q.r.) total plasma nitrate and nitrite concentrations were significantly higher in mice consuming quercetin on day 30 (quercetin, $n = 12$; controls, $n = 12$). **c,d** Median values, interquartile ranges and ranges are denoted by horizontal bars, boxes and error bars respectively. *$P = 0.021$ (Mann–Whitney *U* test). For *P* values, see main text.

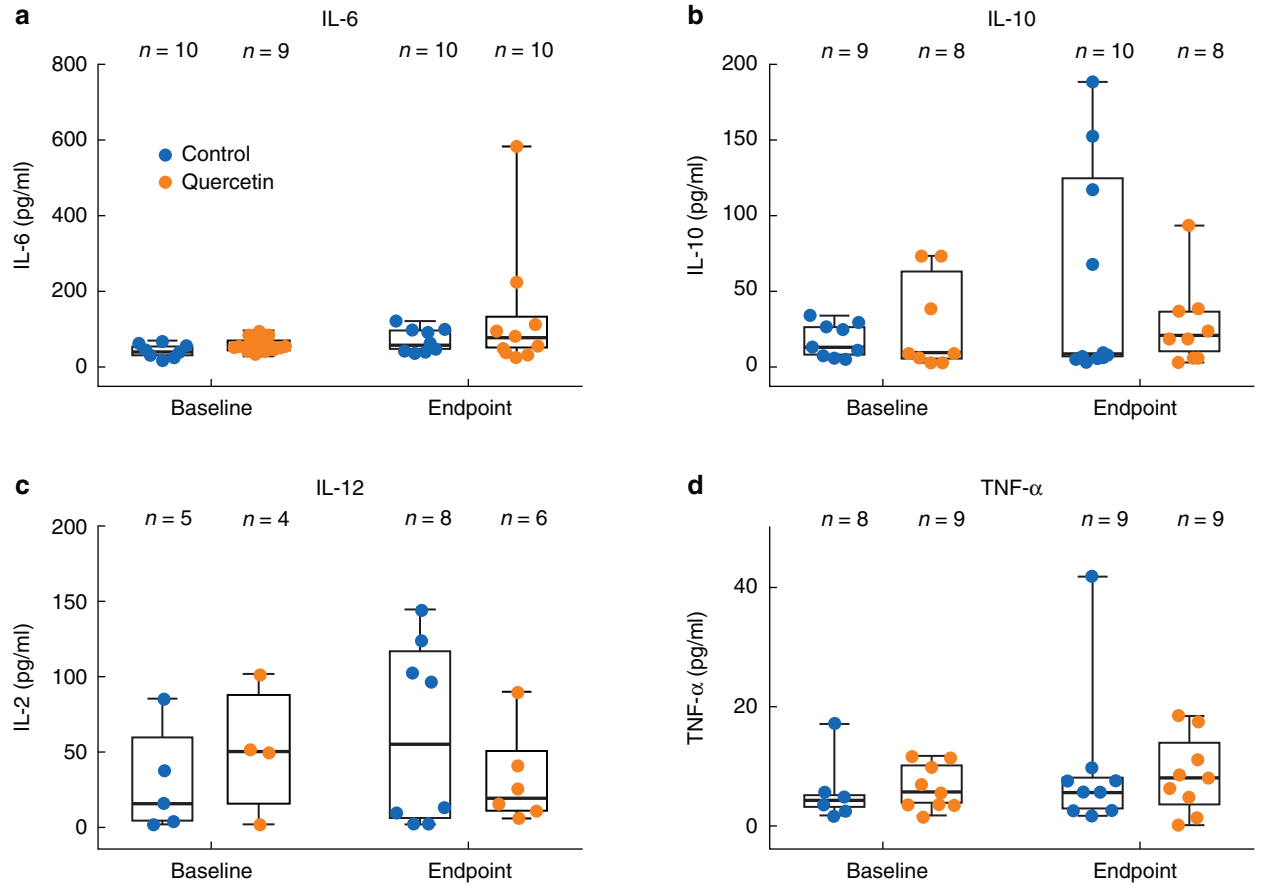

Fig. 3 Plasma cytokine concentrations at baseline before induction of hind-limb ischaemia or quercetin administration and at the endpoint after 4 weeks of quercetin

a Plasma interleukin (IL) 6, **b** IL-10, **c** IL-12, and **d** tumour necrosis factor (TNF) α concentrations. Values are median (i.q.r.). Median values, interquartile ranges and ranges are denoted by horizontal bars, boxes and error bars respectively. For *P* values, see main text.

no significant difference in the ratio of withdrawal force after pressure application between control (median 0.8 (i.q.r. 0.6–1.2)) and quercetin (0.9 (0.6–1.2)) groups on day 48 ($P = 0.866$. Mann–Whitney *U* test) (*Fig. 1b*),. Mean(s.e.m.) velocity (43.3(1.9) *versus* 45.4(1.8) mm/s; $P = 0.412$, unpaired 2-tailed *t* test) and distance travelled (42.8(2.1) *versus* 47.0(1.9) mm; $P = 0.151$, unpaired 2-tailed *t* test) in an open field test were also not significantly different between control and quercetin groups on day 44 (*Fig. 1c,d*).

Hind-limb perfusion ratios decreased following the two-stage HLI model compared with baseline in mice subsequently allocated control (mean(s.e.m.) 1.03(0.04) *versus* 0.55(0.02)) or quercetin (1.05(0.04) *versus* 0.57(0.03)) diets ($P < 0.001$). The degree of HLI did not significantly differ between mice allocated control (0.42(0.02), 0.49(0.03), 0.43(0.03)) and quercetin (0.40(0.03), 0.45(0.03), 0.41(0.03)) diets on days 28, 35 and 49 ($P = 0.954$) (*Fig. 2a,b*). Total plasma nitrate and nitrite concentration was not significantly different between mice allocated control (median 196.6 (i.q.r. 70.4–359.4) mmol/l) and quercetin (92.3 (32.5–326.8) mmol/l) diets on day 0 ($P = 0.272$, Mann–Whitney *U* test) (*Fig. 2c*). Mice that consumed quercetin had significantly greater total plasma nitrate and nitrite concentrations than controls 30 days after surgery (173.4 (104.9–195.7) *versus* 94.6 (51.5–127.2) mmol/l; $P = 0.021$, Mann–Whitney *U* test) (*Fig. 2d*).

There was no significant difference between control and quercetin groups at the study endpoint in plasma TNF-α (median 5.6 (i.q.r. 2.6–8.6) *versus* 8.0 (3.3–14.4) pg/ml respectively; $P = 0.619$, Mann–Whitney *U* test), IL-6 (54.9 (41.2–100.2) *versus* 75.5 (45.8–139.6) pg/ml; $P = 0.565$, Mann–Whitney *U* test), IL-10 (6.4 (3.3–125.8) *versus* 18.9 (6.5–36.4) pg/ml; $P = 0.704$, Mann–Whitney *U* test) or IL-12 (54.6 (3.4–118.6) *versus* 19.3 (8.6–52.3) pg/ml; $P = 0.724$, Mann–Whitney *U* test) (*Fig. 3*).

Discussion

This study found that administration of quercetin after induction of HLI did not improve exercise performance or increase physical activity in a mouse model designed to simulate the physical performance impairment caused by PAD. It was reported previously[6] that administration of isoquercitrin conjugated to glucose moieties (a form of bioavailable quercetin) to mice improved hind-limb blood supply when started 2 weeks before induction of acute HLI. Conversely, the present study found that administration of quercetin after HLI induction did not improve limb perfusion.

Previous mouse studies[7,13,21–24] reported that a dose of 185 mg/kg daily of quercetin, as used in the present study, was effective at improving exercise performance, reducing muscle atrophy, and reducing inflammation. This dose was also estimated to equate to 1000 mg/day in human subjects based on body surface area calculations[25]. A dose of 1000 mg/day has been tolerated in human subjects for up to 8 weeks, with no reported

side-effects, and beneficial effects on exercise performance in healthy subjects have been reported at this dose[26,27]. In the present study, administration of quercetin increased total plasma nitrates (an indicator of nitric oxide), suggesting the dose of quercetin was adequate. Nitric oxide has been critically implicated in improving blood flow and increasing angiogenesis, and is the suggested mechanism by which quercetin has improved hind-limb blood supply in previous studies[6]. Based on these data, quercetin would be expected to promote angiogenesis and thus limb blood supply, but in the present study quercetin had no effect on limb perfusion.

This study used old mice that had dyslipidaemia and sustained HLI in order to model human PAD. Old mice are less responsive to interventions that stimulate arteriogenesis, angiogenesis, and mitochondrial biogenesis[28]. The two-stage surgical model used in the present study also limits changes in shear stress due to gradual occlusion, and limits the collateral circulation that usually forms quickly after acute HLI[14,29]. This may explain why quercetin did not improve exercise performance and blood flow in the present study, in contrast to previously reported studies utilizing other mouse models[13].

Limb pain is a common symptom in patients with PAD, but difficult to assess in animal models. Quercetin has been reported previously[9] to reduce pain from acetic acid injection; however, the effects of quercetin on pain have not been evaluated previously in HLI models. The present study assessed the effects of quercetin on hind-limb hypersensitivity to mechanical pressure and found no effect. Furthermore, quercetin administered in the diet did not appear to alter plasma IL-6, IL-10, IL-12 or TNF-α concentrations, although the sample sizes were small for these comparisons. The findings suggest that quercetin is unlikely to be beneficial for pain secondary to ischaemia.

This study had a number of strengths and limitations. Strengths were the inclusion of key design features, such as adequate sample sizes and use of a clinically relevant model. Quercetin was added to the diet to mimic human consumption through the diet as opposed to direct administration through gavage. Bodyweight data suggested that mice in both groups consumed diet equally. Supplementation may be modelled through gavage in mice, and this route of administration may have shown better efficacy. Quercetin was administered after sustained HLI had been established, in order to reflect what occurs clinically, as patients seek treatment once symptoms have developed. Quercetin was tested over a 30-day period, and it is possible that a longer duration of administration may have had a different effect. Male mice were used in this study, as the newly developed model of sustained hind-limb ischaemia has not yet been tested in female mice. It is important that future studies use female mice as PAD is of equal burden in women[30].

Acknowledgements

This research was not preregistered with an analysis plan in an independent institutional registry. Request for original data and materials can be made to the corresponding author.
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Disclosure. The authors declare no conflict of interest.

Supplementary material

Supplementary material is available at *BJS Open* online.

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