The flavonols Quercetin and 3', 4'-Dihydroxyflavonol reduce platelet function and delay thrombus formation in a model of type 1 diabetes

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Summary

Flavonols are polyphenolic compounds with reported cardiovascular benefits and antiplatelet properties. We have recently shown that the naturally occurring flavonol quercetin (Que) or the synthetic flavonol 3',4'-dihydroxyflavonol (DiOHF) inhibit platelet function and delay thrombus formation in healthy mice. Diabetes is associated with increased cardiovascular risk, therefore, the aim of this study was to investigate the effect of daily 6 mg/kg intraperitoneal treatment of Que or DiOHF over 7 consecutive days on platelet aggregation, granule exocytosis and ferric chloride-induced carotid artery thrombosis in a mouse model of type 1 diabetes. Vehicle treated diabetic mice showed rapid thrombus formation, and achieved greater than 90% blood flow reduction within the 30 min of recording, while diabetic mice treated with 6 mg/kg of Que or DiOHF maintained blood flow at a significantly higher level at the end of the recording period. Platelets derived from diabetic mice were hyper-aggregable in response to platelet agonist stimulation when compared to the vehicle treated control mice, however, platelet aggregation in diabetic mice was significantly reduced with Que or DiOHF treatment. Treatment with 6 mg/kg of Que or DiOHF significantly inhibited dense granule exocytosis as measured by quinacrine uptake and release in diabetic and control mice, while there was no inhibitory effect on alpha granule exocytosis as measured by P-selectin expression in either diabetic or control mice. These data demonstrate that Que and DiOHF inhibit of platelet function and thrombus formation *in vivo* in a model of diabetes, and suggest a potential clinical role for flavonols as anti-platelet therapy.

Keywords diabetes, flavonol, 3', 4'-Dihydroxyflavonol, platelet, quercetin, thrombosis

Introduction

Diabetes is a growing health problem and it has been estimated that the number of people with diabetes will rise to 439 million by 2030 worldwide [1]. In Australia approximately 4.4% of the total population have been diagnosed with diabetes. In addition to its high prevalence, diabetes costs Australians in excess of $6 billion per year [2, 3]. Hyperglycaemia is often associated with macro-vascular complications [4], presenting as accelerated atherosclerosis leading to coronary, cerebral and peripheral arterial disease. Diabetes is therefore an important independent risk factor for cardiovascular disease (CVD) and a major contributor to cardiovascular events, particularly in Western countries. The incidence of CVD is between 2 and 4 times greater in people with diabetes [5], and people with diabetes have a poorer prognosis [6-8]. Indeed, recent reports from the World Health Organization (WHO) state that 50% of diabetic patients will die from CVD [9]. Thrombotic complications of cardiovascular disease, such as myocardial infarction (MI) and stroke, are responsible for up to 84% of deaths in people with diabetes aged 65 and older [5, 10]. In Australia it was reported that 65% of all CVD deaths occur in people with diabetes or impaired glucose metabolism [11]. Therefore it is vital develop new treatments to reduce diabetes-induced CVD.

Antithrombotic agents are regularly used for both the primary and secondary prevention of cardiovascular events in diabetes [12, 13], but there is growing data to suggest inadequate cardiovascular protection by these agents [14]. A meta-analysis of randomized trials showed no significant benefit of aspirin in reducing clinical ischaemic events in people with diabetes [15-17], and therefore alternative therapies are required. Over the last decade many naturally occurring food components have been investigated for their health benefits, particularly, in reducing diabetes-induced complications [18]. Flavonols are one of the most common food derived polyphenolic compounds, which are associated with vascular beneficial properties [19].

The Rotterdam study showed a reduction in the occurrence of myocardial infarction with increased flavonol intake [20]. Furthermore, previous studies have shown that ingestion of flavonol rich foods and beverages reduce platelet aggregation [21-24]. Que is ubiquitously found in a variety of fruits and vegetables, and is one of the most abundant naturally occurring flavonols, [25]. We have recently shown that Que and DiOHF a synthetic flavonol of similar structure to Que, inhibit human platelet aggregation and dense granule exocytosis

in a concentration dependant manner [26]. Furthermore, we showed that the administration of Que or DiOHF at 6 mg/kg for seven consecutive days inhibits platelet function and reduces thrombus formation in a mouse model of platelet mediated arterial thrombosis [27].

It has been reported in animal models of diabetes that treatment with Que restores endothelial function [28] and reduces pancreatic β-cell injury [29], systolic blood pressure, plasma lipids and plasma glucose levels [30, 31]. DiOHF also has demonstrated ability to prevent diabetes-induced endothelial dysfunction [32], increase nitric oxide activity [33] and to restore endothelium dependent relaxation [34].Therefore, the aim of this study was to investigate the effects of Que or DiOHF on arterial thrombus formation and platelet function ex vivo in an animal model of diabetes.

Materials and Methods

Animals

All experimental procedures performed in this study were approved by the Animal Experimentation Ethics Committee of RMIT University and were conducted in accordance with the guidelines of the National Health and Medical Research Council of Australia for the care and use of animals for scientific purposes.

Induction of diabetes and administration of investigational agents

Diabetes was induced according to previously described methods [35, 36] with minor modifications. Briefly, diabetes was developed in C57BL/6 mice following a series of five intraperitoneal i.p. injections with streptozotocin (60 mg/kg) dissolved in citrate buffer. Diabetes was confirmed when the blood glucose level was > 13 mmol/L. Mice that did not develop diabetes within 4 days of the last STZ injection were given a second series of five STZ injections. Where blood glucose exceeded >30 mmol/L insulin was administered at 0.1 to 0.2 U/mouse i.p. 2-3 times a week. Control mice received an equal amount of citrate buffer alone. Following 8 weeks of STZ-induced diabetes or an equivalent control period, mice were randomised to receive Que (6 mg/kg), DiOHF (6 mg/kg) or vehicle (0.5% DMSO plus 20% polyethelene glycol (PEG) and saline), via i.p. injection daily for 7 days. Experimental procedures and blood collection were performed 24 h following the last treatment.

Ferric chloride induced carotid injury

Ferric chloride-induced arterial injury was performed as previously described [37]. Briefly, mice were anaesthetised with ketamine and xylazine (200: 10 mg/kg) by IP injection. A midline cervical incision was made and the carotid artery exposed. A laser Doppler flow probe (Moor Instruments, UK) was placed adjacent to the carotid artery to measure baseline blood flow. After baseline blood flow was established, a 2 x 4 mm filter paper saturated with 20% ferric chloride was applied to the carotid artery on the adventitial surface of the carotid artery for 4 min. Following the removal of the filter paper blow flow through the carotid artery was monitored for 30 min, or until a 95% reduction in blood flow was reached. At the end of each experiment, and whilst the mouse was under deep anaesthesia, it was euthanized by cervical dislocation.

Sample preparation for platelet aggregation and dense granule exocytosis

In separate mice, fresh whole blood was collected via cardiac puncture and placed into tubes containing 100 µl of 3.2% (w/v) sodium citrate. Platelet rich plasma (PRP) was obtained by centrifugation at 200 x g for 15 min at room temperature with no brake. Platelet poor plasma (PPP) was obtained by centrifugation of the remaining blood at 1200 x g for 15 min at room temperature. Platelet count was performed using a Beckman Coulter Ac.T 5 Haematology analyser (Coulter Corporation, USA). Platelet count was normalised in all treatment groups to 100 x 10^9 /L in Ringer citrate dextrose (RCD) buffer, pH 7.4 (108 mM NaCl, 38 mM KCl,1.7 mM $NaHCO_3$, 21.2 mM sodium citrate, 27.8 mM glucose, and 1.1 mM $MgCl_2 \cdot 6\ H_2O$, with pH adjusted to 7.4).

Platelet aggregation

Platelet aggregation was measured by turbidimetric aggregometry using a Chrono-log 700 aggregometer (Chrono-log Corp, USA) [38]. In brief, PRP aggregation was performed in the presence of 100 µg / mL fibrinogen and 1 mM $CaCl_2$ at 37°C with constant stirring (1000x rpm). Platelet aggregation baseline was set against murine PPP diluted 1: 2 in RCD buffer. Platelet aggregation was stimulated using 250 µM of the protease-activated receptor 4 (PAR 4) agonist peptide H-Ala - Tyr - Pro - Gly - Lys - Phe -NH2 (AYPGKF-NH2) (GL chemicals, China). The maximal aggregation amplitude over a 9 minute period was recorded.

Assessment of granule exocytosis

Dense granule exocytosis was measured by flow cytometry using fluorescent quinacrine uptake and AYPGKF-NH2 induced release. Aliquots of mouse PRP were incubated with 100 µM quinacrine at 37°C for 20 min in the dark. The platelets were then washed using RCD buffer by centrifugation at 500 x g for 10 min with no brake at room temperature and resuspended in RCD buffer. Aliquots were incubated with 250 µM AYPGKF-NH2 at 37°C for 5 min in the dark. The reaction was stopped by 1:12 dilution of RCD, and immediately analysed by flow cytometry. Alpha granule exocytosis was measured in separate aliquots of PRP by incubating with anti-mouse CD62P-PE (BD Pharmingen, USA) and 250 µM AYPGKF-NH2 at 37°C for 30 min. Flow cytometric analysis was performed using a FACSCanto II flow cytometer (BD Biosciences, USA) with excitation at 488 nm and quinacrine fluorescent emission collected in the range 515 – 545 nm and CD62P-PE fluorescent emission collected in the range 564 – 606 nm. Staining in separate aliquots

prevented the need for compensation of spectral overlap. 10,000 individual platelets were analysed. Dense granule exocytosis was recorded as the percentage of decrease in 515 – 545 nm fluorescence intensity when compared to unstimulated platelets, while alpha granule exocytosis was recorded as CD62P mean fluorescent intensity (MFI).

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). Comparisons between test samples and control were performed using one-way ANOVA test with Dunnett's test, for post hoc comparisons. An unpaired Student's t-test was performed to compare diabetic and non-diabetic platelet aggregation and dense granule exocytosis.

Results

Weight and plasma glucose levels

Over the 8 week period, the STZ-treated mice gained less weight compared to non-diabetic control (Figure 1A). Similarly, the blood glucose levels in STZ-treated mice were significantly higher than non-diabetic mice (Figure 1B).

Effect of Que and DiOHF on $FeCl_3$ induced arterial thrombosis

Diabetic mice showed rapid thrombus formation when compared to the non-diabetic mice. In vehicle treated diabetic mice, carotid blood flow was reduced to 50±17% of baseline 5 min after application of $FeCl_3$ (Fig 2A), while vehicle treated control mice maintained greater than 90% blood flow at the same time (94±7% flow, $p < 0.05$ vs. diabetic vehicle treated mice, Fig 2B). However, at 15 min after application of $FeCl_3$ both diabetic and non-diabetic vehicle treated mice had near complete vessel occlusion (1±1% flow vs 6±1% flow, respectively, $p < 0.05$. between diabetic and non-diabetic mice, Figs 2 A&B). There was no blood flow at 30 min as there was total occlusion in vehicle treated diabetic and non-diabetic mice.

Treatment of diabetic mice with 6 mg/kg of Que or DiOHF significantly improved blood flow in the carotid artery at 5 min (Que 94 ± 5% flow, DiOHF 93±7% flow, $p < 0.05$ vs. diabetic vehicle treated mice, Fig 2A). At 15 min flavonol treated diabetic mice maintained significant blood flow (Que 61±15% flow, DiOHF 83±18% flow, $p < 0.05$ vs. diabetic vehicle treated mice, Fig 2A). Similarly, blood flow at 15 min was well maintained in non-diabetic mice treated with 6 mg/kg of either Que or DiOHF (Que 91±6% flow, DiOHF 70±18% flow, $p < 0.05$ vs. non-diabetic vehicle treated mice, Fig 2B). Blood flow at 30 min after $FeCl_3$ injury was well maintained in diabetic mice treated with the flavonols (Que 48±21% flow, DiOHF 52±19% flow, Fig 2A).

In contrast, when non-diabetic mice were treated with the flavonols, both Que and DiOHF maintained blood flow 30 min after $FeCl_3$ injury, however Que maintained higher blood flow when compared to DiOHF (Que 75±18% flow, DiOHF 53±26% flow, Fig 2B). When blood flow in both diabetic and non-diabetic mice was expressed as the area under the curve flavonol treated mice showed significantly greater blood flow than vehicle treated diabetic and non-diabetic mice, see Fig 3.

Effect of Que and DiOHF on platelet aggregation

Platelets derived from diabetic mice showed a higher level of aggregation to AYPGKF-NH_2 stimulation when compared to the vehicle treated control mice (102±9% diabetic platelets vs. 78±2% control platelets, $p < 0.05$). Platelet hyper-aggregability in diabetic mice was significantly reduced following 7 day treatment with Que or DiOHF (64±7and 70±9%, respectively, $p < 0.05$ vs. vehicle, Fig 4A). In control mice Que or DiOHF treatments, as expected, significantly reduced platelet aggregation (Que 53±6% and DiOHF 53±10%, $p < 0.05$ vs. vehicle, Fig 4B).

Effect of Que and DiOHF on granule exocytosis

There was no significant difference in dense granule exocytosis, as measured by quinacrine release, between vehicle treated diabetic and non-diabetic mice in response to AYPGKF-NH_2 stimulation (51±4% vs. 58±3%, $p > 0.05$). Treatment with 6 mg/kg of Que or DiOHF significantly inhibited dense granule exocytosis in diabetic (Que 34±4% and DiOHF 34±3%, $p < 0.05$ vs. vehicle, Fig 5A1), and non-diabetic mice (Que 48± 3% and DiOHF 46±2%, $p < 0.05$ vs. vehicle, Fig 5A2).

Treatment with 6 mg/kg of Que or DiOHF did not produce an inhibitory effect on alpha granule exocytosis as measured by P-selectin expression induced by AYPGKF-NH_2 in either diabetic (Que 2850±334 fluorescence intensity and DiOHF 3328±420 fluorescence intensity vs. vehicle 3296±619 fluorescence intensity, $p > 0.05$, Fig 5B1) or non-diabetic mice (Que 2671±463 fluorescence intensity and DiOHF 3306±694 fluorescence intensity vs. vehicle 3257±297 fluorescence intensity, $p > 0.05$, Fig 5B2).

Discussion

In this study we have shown that i.p. treatment with Que or DiOHF for seven consecutive days delay thrombus formation in a model of platelet mediated thrombosis in type 1 diabetic mice. Furthermore, we show the delay in thrombus formation is at least in part due to the inhibition of platelet aggregation and dense granule exocytosis.

Platelet hyper-sensitivity plays a major role in cardiovascular complications of diabetes [39, 40], therefore the aim of the study was to investigate the effects of Que and DiOHF on platelet function and thrombus formation. In this study STZ was used to induce type 1 diabetes in C57BL/6 mice. $FeCl_3$ was used to induce arterial thrombosis in the carotid artery. $FeCl_3$ induces platelet mediated thrombus formation by damaging the endothelial lining, causing RBC haemolysis and haemoglobin oxidation. This leads to ROS production, causing further endothelial damage, resulting in increased platelet activation and adhesion at the site of injury [41].

Diabetic mice showed rapid thrombus formation when compared to healthy mice. Indeed, thrombus formation in diabetic mice commenced prior to the removal of the $FeCl_3$ strip, and when the $FeCl_3$ strip was removed blood flow had fallen below the base line. In contrast, non-diabetic mice maintained 100% blood flow during and after $FeCl_3$ application. Furthermore, at 5 min after $FeCl_3$ application blood flow in diabetic mice was less than 50% of baseline, whilst blood flow in control mice was close to pre-injury levels. This supports previous observations that in mice, hyperglycaemia accelerates thrombus formation when induced by $FeCl_3$ [42], and suggests that diabetes accelerates thrombus formation, but there is no difference in the magnitude of thrombus formation over 30 min. The accelerated thrombus generation following arterial injury in diabetic mice observed in this study corresponded to enhanced agonist stimulated platelet aggregation. It is well established that impairment of endothelial function is associated with diabetes, and is likely to contribute to the accelerated thrombus generation in diabetics. This may be due to the reduced production of NO [43] and other vasorelaxant and antithrombotic agents such as prostacyclin from the endothelial cells [44], in addition to the enhanced platelet aggregation associated with this disease state.

Treatment with 6 mg/kg of either Que or DiOHF significantly improved blood flow and delayed thrombus formation in diabetic mice. Indeed, blood flow in flavonol treated diabetic mice was maintained to near 100% flow 5 min after $FeCl_3$ application, and there was no

significant difference in blood flow between diabetic and non-diabetic mice at that time of recording. In addition, full vessel occlusion did not occur at 30 min after $FeCl_3$ injury.

In our diabetic model, platelets were found to be hyper-aggregable in response to AYPGKF-NH_2 when compared to non-diabetic mice, which is in agreement with previous observations that diabetic animals show enhanced platelet aggregation [45-47], and also corresponds with the rapid formation of a platelet mediated thrombus. On the other hand, alpha and dense granule exocytosis was not significantly different between diabetic and non-diabetic mice. Treatment with 6 mg/kg of Que or DiOHF for seven consecutive days significantly reduced platelet aggregation, and inhibited dense, but not alpha, granule exocytosis in response to AYPGKF-NH_2. Platelet aggregation was reduced by more than 30% in platelets from diabetic mice and the levels were similar to that of the non-diabetic mice.

Although the delay in thrombus formation observed in this study corresponds to the reduction of platelet aggregation and dense granule exocytosis, it is likely that the effect of these flavonols on endothelial function also contributed to the overall vascular benefits achieved. Que and DiOHF have been reported to produce potent vasoprotective effects on both healthy and diabetic models. Que has also been demonstrated to exert beneficial vascular effects in a range of pathological conditions including diabetes [48-50]. Indeed, it has been shown to restore endothelial relaxation in diabetes in response to acetylcholine, increase NO, neutralise free radicals and reduce oxidative damage in STZ treated rats [28, 51, 52]. Que has also been reported to inhibit lipid peroxidation via inhibition of xanthine oxidase [53].

This study demonstrates that 6 mg/kg of the flavonols is capable of delaying thrombus formation in diabetes, which is consistent with our previous studies [32-34] reporting daily i.p. treatments of 5 mg/kg of flavonols for 7 days, reduced O_2^- formation and prevented endothelial dysfunction in diabetes. Freedman et al [23] showed reduced platelet aggregation and enhanced NO production following the consumption of 7 mL/kg per day of purple grape juice for 14 days.

This study provides evidence that naturally occurring and synthetic flavonols are able to produce beneficial effects in the vasculature in the context of a diabetic hyper-thrombotic state. Therefore, further investigation of supplementation and optimal doses that are capable of producing beneficial effects in humans are warranted,

An unexpected finding of this study was an absence of any inhibition of alpha granule exocytosis by the flavonols, whereas inhibition of dense granule exocytosis was observed. Platelet alpha granule secretion occurs more readily than dense granule secretion, however the mechanisms leading to membrane fusion and exocytosis of the two granule types have generally been assumed to be similar [54, 55]. By contrast studies have shown that aspirin is capable of inhibiting ADP-induced serotonin release (a dense granule component) whilst P-selectin expression is unaffected [55], suggesting potential for selective inhibition of exocytosis by the different granule types. The results obtained in the current study suggest a similar mechanism of selective inhibition of dense granule exocytosis whilst alpha granule exocytosis is maintained. Further investigation, including investigation of different concentrations of the flavonols, and exocytosis induced by different chemical agonists, is warranted before conclusions can be drawn from this interesting observation. Given the ability of these flavonols to restore blood flow and platelet aggregation in diabetic mice to normal levels, more studies are warranted to determine optimal doses, and also structural modifications to these compounds that might increase the antithrombotic potency.

Limitations

This study demonstrates improved blood flow and reduced platelet hyper-sensitivity in type 1 diabetes, and although the pathology in type 1 and 2 diabetes is similar, these improvements also need to be demonstrated in type 2 diabetes. Furthermore, reduced platelet aggregation might be associated with increased bleeding risks, and although significant bleeding was not observed, appropriate studies investigating the effect of these flavonols on bleeding times are warranted.

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Figure 1. STZ-induced diabetes in C57BL/6 mice. A) Weight of diabetic and non-diabetic mice over the 8 week period, and B) Plasma glucose levels of diabetic and non-diabetic mice during the 8 week period, n= 35 each group.

Figure 2. Mean arterial blood flow expressed as a percentage of baseline. A) Carotid blood flow in diabetic mice treated with 6 mg/kg Que, 6 mg/kg DiOHF or vehicle control following ferric chloride induced arterial injury. (B) Carotid blood flow in non-diabetic mice treated with 6 mg/kg Que, 6 mg/kg DiOHF or vehicle control following ferric chloride induced arterial injury, n= 4 for each treatment group. For clarity, flow is shown as the mean without sem.

Figure 3. Carotid artery blood flow expressed as the area under the curve (AUC) over 30 min for the mice treated with 6 mg/kg Que, 6 mg/kg DiOHF or vehicle control following ferric chloride induced arterial injury. (A) Diabetic mice. (B) Non-diabetic mice. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post test.

Figure 4. Platelet aggregation stimulated with AYPGKF-NH_2 (250 μM). Platelets were obtained from diabetic and non-diabetic mice treated with 6 mg/kg Que (n = 5), DiOHF (n = 5) or vehicle (n = 4). Platelet count was normalised to $100x10^9$/L in all test groups. (A) Diabetic mice. (B) Non-diabetic mice. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post test.

Figure 5. Granule exocytosis stimulated with PAR 4 agonist peptide (250 μM). Platelets were obtained from diabetic and non-diabetic mice treated with 6 mg/kg Que (n=6), DiOHF (n=6) or vehicle (n=6). Platelet count was normalised to $100x10^9$/L in all test groups. (A1) Dense granule exocytosis as measured by quinacrin uptake and release in diabetic mice. (A2) Dense granule exocytosis as measured by quinacrin uptake and release in non-diabetic mice. (B1) Alpha granule exocytosis as measured by the mean fluorescence intensity of P-Selectin expression in diabetic mice. (B1) Alpha granule exocytosis as measured by the mean fluorescence intensity of P-Selectin expression in non-diabetic mice. * $P < 0.05$ vs vehicle. Mean ± SEM. One way ANOVA with Dunnett's post test.

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