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Blueberry juice causes potent relaxation of rat aortic rings *via* the activation of potassium channels and the H_2S pathway

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The objective of this study was to investigate the *in vitro* effects of blueberry juice on healthy rat aortic rings, and to explore the roles of potassium channels and of the hydrogen sulphide (H_2S) pathway in mediating the effects of blueberry juice. Firstly, the antioxidant capacity of blueberry juice was compared to other popular juice drinks using the Folin–Ciocalteu and the DPPH assays. Blueberry juice had significantly higher total polyphenol content than any of the other drinks studied ($p < 0.01$). The effect of blueberry juice on noradrenaline-contracted aortic rings was then observed, and the juice caused significant inhibition of noradrenaline-induced contractions ($p < 0.01$). Voltage-gated potassium channel (Kv) blockers 4-aminopyridine (1 mM) and 3,4-diaminopyridine (1 mM), as well as the cystathionine γ-lysase (CSE) inhibitor D,L-propargylglycine (2 mM) were then utilised to elucidate the role of Kv channels and the CSE/H_2S pathway. Kv channel blocker 3,4-diaminopyridine caused significant blockade at 1/100 and 1/50 dilutions of juice ($p < 0.01$), whilst 4-aminopyridine caused significant blockade of the 1/100 dilution of blueberry juice ($p < 0.05$). In addition, D,L-propargylglycine potently inhibited the effect of 1/100 and 1/50 dilutions of blueberry juice ($p < 0.01$). This study indicates that blueberry juice has potent vasorelaxing properties, and thus may be a useful dietary agent for the prevention and treatment of hypertension. This study also provides strong evidence that Kv channels and the CSE/H_2S pathway may be responsible, at least in part, for mediating the effects of blueberry juice.

1 Introduction

Along with other berries such as cranberry, blueberries can be biologically classified into the genus *Vaccinium*, which means a berry-bearing shrub. It was originally a native North American fruit but it is widely grown today, and there are many different varieties. Commercially, the most important varieties are probably *Vaccinium corymbosum* (highbush) and *Vaccinium angustifolium* (lowbush).[1] Blueberries are known to be particularly rich in anthocyanins, including malvidin-3-*O*-galactoside and delphinidin-3-*O*-galactoside, as well as flavanols and other polyphenols. In terms of antioxidant content, they have outperformed many well-reputed fruits and vegetables in the ORAC (oxygen radical absorbance capacity) and other assays.[2–4]

Some animal studies have previously shown that consumption of blueberries may have a role in the treatment of hypertension, as well as other cardiovascular diseases.[4–13] Blueberries have also been found to have beneficial effects for men and women with obesity and metabolic syndrome, conditions which are closely linked to hypertension.[14,15] However, only a small number of studies have investigated the effects and the mechanisms of action of blueberries and blueberry juice in healthy vascular tissue.[13,16] In relation to hypertension, the effects of blueberries and blueberry juice in health is of great importance, as it is well known that hypertension, in particular age-related hypertension, can often be prevented by healthy diet and lifestyle.[17]

Regarding the mechanism of action of fruits and juices in vascular tissue, any vasorelaxant effect is often attributed to an increased availability of nitric oxide (NO) from the endothelium.[18–22] However, some studies have implicated a role for K^+ channels in mediating the vasorelaxant effects of polyphenols.[23–25] Given the crucial role that potassium channels play in vascular smooth muscle,[26] we hypothesise that any effect of blueberry juice on the vascular endothelium may be mediated, at least in part, by activation of K^+ channels. Indeed, some studies have linked poor dietary habits to dysregulation of potassium channels and associated hypertension.[27,28] Closely associated with the activity of K^+ channels, is the CSE/H_2S (cystathionine γ-lysase/hydrogen sulphide) pathway. Whilst the NO pathway has conventionally been accredited with a role in mediating the vasorelaxant effects of fruit juices, very little attention has been given to H_2S, another gasotransmitter with vasodilator properties. H_2S has been shown to mediate its effects *via* the activation of K^+ channels.[29–31] Given the evidence

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that H_2S causes arterial relaxation,[29,30,32–40] it is of huge interest to investigate the role of this pathway, as well as the role of K^+ channels in mediating any effects of blueberry juice.

The objectives of this study therefore were to (a) compare the antioxidant status of blueberry juice with some other well-known juice drinks, (b) to determine the *in vitro* effects of blueberry juice on healthy vascular tissue from the rat and (c) to investigate the roles of the voltage-gated K^+ (Kv) channels and the CSE/H_2S pathway in mediating any effects of blueberry juice on rat aortic tissue.

2 Materials and methods

2.1 Reagents

Acetylcholine bromide, 4-aminopyridine (4-AP), 3,4-diaminopyridine (3,4-diAP) and D,L-propargylglycine (PPG) were purchased from Sigma-Aldrich. Noradrenaline was purchased from Abbott Laboratories Ltd. Pure Biona blueberry, cranberry, pomegranate and red grape juices were purchased locally from Evergreen Health Store. Blackcurrant cordial used was Ribena Toothkind™ and was purchased from a local Tesco outlet. Folin–Ciocalteu (F–C) reagent, 1,1-diphenyl-2-picrahydrazyl (DPPH), ascorbic acid and gallic acid were purchased from Sigma Aldrich.

The blueberry juice utilised in this study is Biona Organic Blueberry Superjuice. According to the manufacturers (Windmill Organics Ltd., Surrey, U.K.), approximately 450 g blueberries are used to produce one 330 ml bottle of juice. The fruit is mashed up, and heated to the optimum processing temperature before undergoing a grinding stage. It then receives enzymatic treatment with temperature control in place. Pectin drawdown is checked and a second enzymatic treatment is then applied if necessary. The juice is pressed, and then bottled.

2.2 The antioxidant capacity of blueberry juice and other juice drinks

2.2.1 Folin–Ciocalteu (F–C) microplate assay. The assay used was modified from the high throughput microplate method described by Magalhães *et al.*[32] For each assay, a standard curve of 0–100 mg L^{-1} gallic acid was created. Juice beverages were diluted within the range of 1/10 000 to neat in order to find the optimal concentrations for the assays. For each assay, the results from at least 3 linear dilutions of each juice were averaged. 50 μl standard or diluted juice sample and 50 μl of F–C reagent (diluted 1 : 2 (v/v) with water) were added to each well of a 96-well plate. Each standard/sample was done in triplicate. 100 μl of 6% (w/v) Na_2CO_3 was added to each well and incubated in the dark for 120 minutes. The absorbance was read on a Victor 3 1420 plate reader at 690 nm. Total Polyphenol Content (TPC) for each juice was expressed as grams of gallic acid equivalents (GAE) per litre of juice drink.

2.2.2 DPPH assay. The free radical 1,1-diphenyl-2-picrahydrazyl (DPPH) was used to assess the radical scavenging activity of the juice drinks. The assay was modified from the assay described by Gorinstein *et al.*[33] Standards in the range of 0–100 μM ascorbic acid were used. Samples and standards were prepared in Tris–HCl buffer. 1 ml sample/standard was added to each cuvette, followed by 2 ml DPPH (200 μM, made up in methanol). The cuvettes were shaken vigorously and incubated in the dark for 30 minutes, before being read at 517 nm in a spectrophotometer (Pharmacia Biotech Ultraspec 4000). Radical scavenging activity (RSA) of ascorbic acid was calculated as a percentage using the following equation:

$$\%\mathrm{RSA} = ((A_o - A)/A_o) \times 100$$

where A_o is the optical density (OD) in the absence of any antioxidant, and A is the OD in the presence of the standard ascorbic acid.

A standard curve of ascorbic acid was created and the radical scavenging activity of each of the juice drinks was then expressed as ascorbic acid equivalents.

2.3 The effects of blueberry juice on rat aortic rings and the mechanism of action

2.3.1 Krebs solution. Krebs solution (pH 7.4) was prepared by dissolving of NaCl (100 mM), KCl (4 mM), $MgSO_4$ (1.5 mM), $NaHPO_4$ (1.5 mM), $NaHCO_3$ (25 mM), $NaCOO_3$ (20 mM) and glucose (10 mM) in distilled water. The solution was heated to 37 °C in a water bath while being constantly bubbled with 95% O_2 and 5% CO_2. $CaCl_2$ (1.8 mM) was added following 20 min of bubbling. The solution was maintained at these conditions daily.

2.3.2 Preparation of rat aorta. Animal sacrifice and tissue harvesting were performed in compliance with the NUI Galway Animal Care and Research Ethics Committee procedures. Animals were cared for in accordance with the Code of Good Practice in Animal Research and were sacrificed humanely. Adult Sprague Dawley rats weighing approximately 250 g were sacrificed by carbon dioxide asphyxiation. The thoracic aorta was carefully removed and transferred immediately into Krebs. The aorta was cleaned of connective tissue and fat, and then cut into ring segments 2–3 mm in length, and care was taken to avoid any damage to the endothelium. Using a syringe, the lumen was flushed through with Krebs solution to remove any clots.

2.3.3 Record of isometric vascular tone. The aortic rings were mounted in a 25 ml organ bath containing Krebs solution by means of two stainless steel wire hooks inserted through the lumen of the ring. Krebs solution was kept at 37 °C, while being continuously bubbled with 95% O_2/5% CO_2. The baseline load placed on the aortic rings was 2 g, and the changes in isometric tension were recorded using a force–displacement transducer connected to a MacLab data acquisition system (AD Instruments). In the first series of experiments, the effect of blueberry juice (BBJ) on noradrenaline (NA)-induced contraction was defined. Aortic rings were contracted with NA (0.1 μM). Endothelial integrity was verified by the presence of a relaxant response to acetylcholine (ACh, 1 μM) on the noradrenaline (NA, 0.1 μM)-contracted vessels. Once a reliable response to NA had been obtained, increasing cumulative concentrations of BBJ (1/1000 to 1/10 dilutions) were added to the bath and each response was recorded for a 10-minute

period. To define the mechanisms by which BBJ relaxes vascular smooth muscle, another series of experiments was performed: the rings were exposed to either 4-AP (1 mM), 3,4-diAP (1 mM) or PPG (2 mM) 15 min prior to exposure to NA, and then vascular relaxation was carried out by cumulative addition of BBJ (1/1000 to 1/10 dilutions) to the tissue bath after NA response reached the plateau. After each test, the aortic rings were washed three times with fresh Krebs solution and allowed to equilibrate for 30 min. The effect of treatment was expressed as a percentage of the NA-induced contractions.

2.4 Statistical analysis

All values were expressed as means ± s.e.m. For the determination of antioxidant capacity, standard curves were constructed using GraphPad Prism™ 3.0. Statistical analyses were performed using SPSS. Significant difference was determined using one or two-way ANOVA or independent *t*-test, using Graph Pad Prism TM 3.0 software. Statistical significance was defined as $p < 0.05$ or $p < 0.01$.

3 Results

3.1 The antioxidant capacity of blueberry juice and other juice drinks

The Total Polyphenol Content (TPC) of blueberry juice (BBJ), as measured by the F–C assay, was on average 3509.06 mg GAE/L (Fig. 1). A one-way ANOVA indicated a highly significant difference in the TPC between the various juice drinks investigated [$F(4, 19) = 460.44$, $p < 0.001$]. Tukey HSD post hoc test revealed that the TPC of BBJ was far higher than that of pomegranate juice ($p < 0.001$), cranberry juice ($p < 0.001$), grape juice ($p < 0.001$), or blackcurrant cordial ($p < 0.001$).

The radical scavenging activity (RSA) of BBJ, as measured by the DPPH assay, was on average equivalent to 46.27 g ascorbic acid per L of juice (Fig. 2). A one-way ANOVA revealed a significant difference in the RSA of the various juice drinks studied [$F(4,10) = 3.76$, $p = 0.041$]. Tukey HSD post hoc test demonstrated that whilst the average RSA of BBJ was higher than that of any of the other drinks studies, statistically it was significantly higher than grape juice only ($p = 0.023$).

3.2 The effect of blueberry juice on noradrenaline-induced contraction of rat aortic rings

Noradrenaline caused an increase in tension of 1.53 g ± 0.25 above baseline ($n = 8$). For each aortic ring, the effect of BBJ was calculated as a percentage of the tension generated by noradrenaline alone. BBJ caused a reduction in NA-induced contractions of rat aortic rings (Fig. 3). The difference between the dilutions was significant according to a one way ANOVA [$F(6, 49) = 10.48$, $p < 0.001$]. Dunnett's 2-tailed post hoc test revealed that 1/250 ($p = 0.002$), 1/100 ($p < 0.001$), 1/50 ($p < 0.001$) and 1/10 ($p < 0.001$) dilutions of juice caused highly significant reductions in NA-induced contraction of rat aortic rings.

3.3 The role of voltage-gated (Kv) potassium channels in mediating the vasorelaxant effects of blueberry juice

An independent *T* test [$t(12) = 2.18$, $p = 0.12$] showed that there was no significant difference between the tension generated by noradrenaline alone (1.53 g ± 0.25 above baseline), and the tension generated by noradrenaline in the presence of 4-AP (0.96 g ± 0.22 above baseline). For each aortic ring, the effect of BBJ in the presence or absence of 4-AP was calculated as a percentage of the NA-induced contraction in that tissue (Fig. 4). A two-way ANOVA demonstrated that there was a significant effect of 4-AP [$F(1, 38) = 5.34$, $p = 0.027$] and a significant interaction between 4-AP and BBJ [$F(2, 38) = 4.26$, $p = 0.023$]. Post hoc analysis revealed that the effect of 1/100 BBJ was

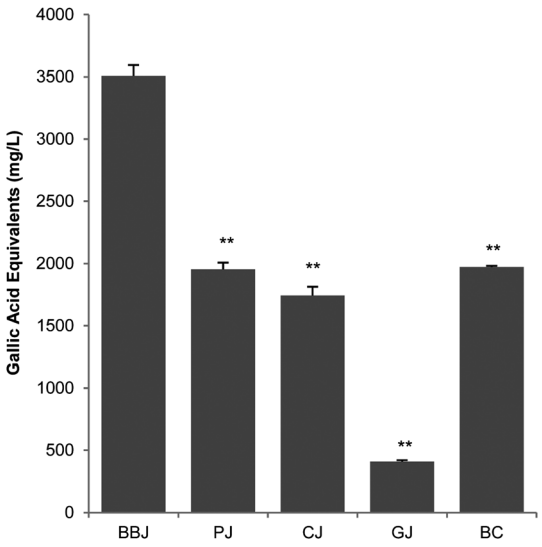

Fig. 1 Total polyphenol content of various juice drinks, measured using the F–C assay. BBJ = blueberry juice; PJ = pomegranate juice; CJ = cranberry juice; GJ = grape juice; BC = blackcurrant cordial. Data represents mean + s.e.m. of 3–6 assays. ** = $p < 0.01$ *vs.* blueberry juice.

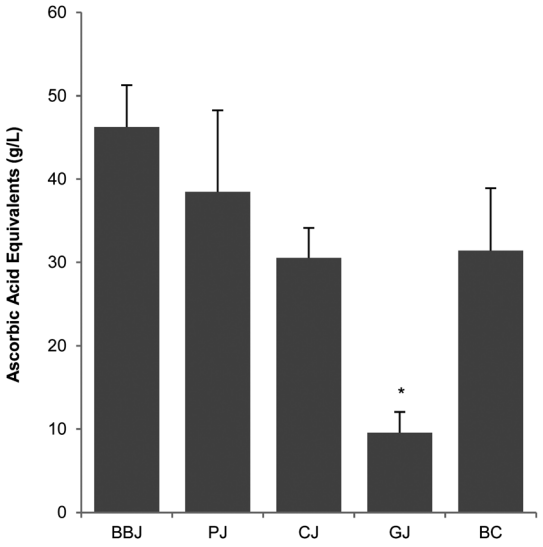

Fig. 2 Radical scavenging activity of various juice drinks, as measured using the DPPH assay. BBJ = blueberry juice; PJ = pomegranate juice; CJ = cranberry juice; GJ = grape juice; BC = blackcurrant cordial. Data represents mean + s.e.m. of 2–4 assays. * = $p < 0.05$ *vs.* blueberry juice.

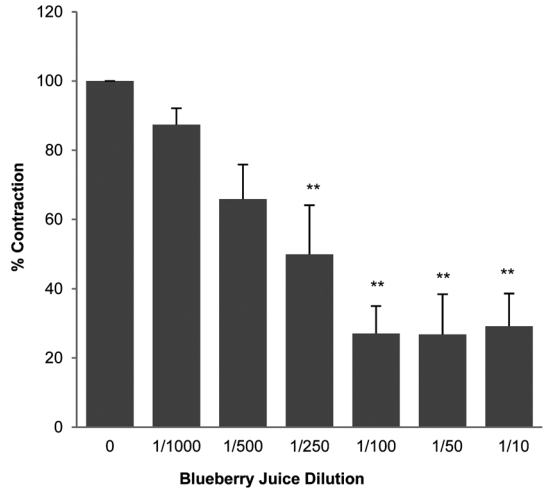

Fig. 3 The effect of blueberry juice on noradrenaline-contracted rat aortic rings. Data shows mean + s.e.m. ($n = 8$) $^{**}p < 0.01$ *vs.* control.

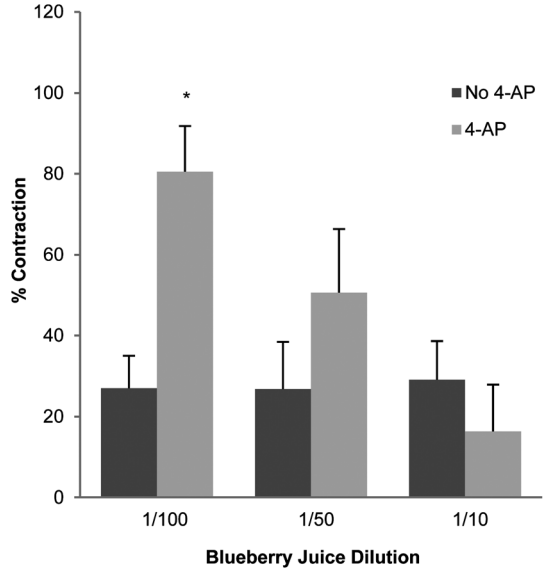

Fig. 4 The effect of 4-aminopyridine (4-AP) on blueberry juice – induced relaxation of rat aortic rings ($N = 7$–8). $^{*}p < 0.05$. *vs.* no 4-AP.

partially blocked by 4-AP ($p = 0.024$ *vs.* BBJ alone). The effect of 1/50 BBJ was also blocked to some extent by 4-AP, but this was not statistically significant. 4-AP had no impact on the relaxant effect of 1/10 dilution of BBJ.

An independent T test [$t(13) = 2.16$, $p = 0.98$] showed that there was no significant difference between the tension generated by noradrenaline alone (1.53 g ± 0.25 above baseline), and the tension generated by noradrenaline in the presence of 3,4-diaminopyridine (1.54 g ± 0.58 above baseline). Similar to above, for each aortic ring, the effect of BBJ in the presence or absence of 3,4-diAP was calculated as a percentage of the NA-induced contraction in that tissue (Fig. 5). 3,4-diAP was observed to be a very potent blocker of the relaxant effects of BBJ. A two-way ANOVA demonstrated that there was a significant effect of 3,4-diAP [$F(1, 44) = 47.39$, $p < 0.001$] and a

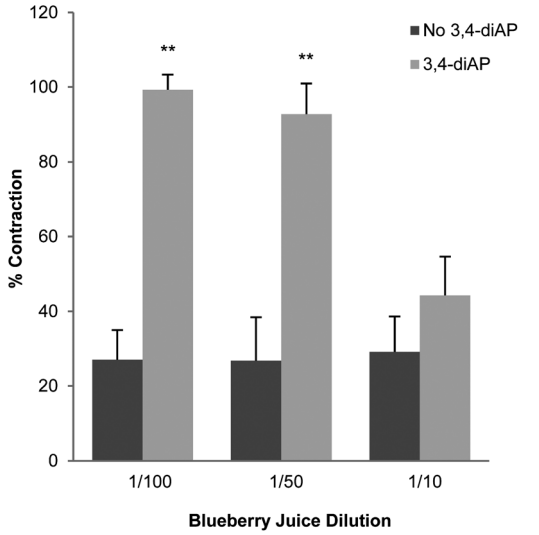

Fig. 5 The effect of 3,4-diaminopyridine (3,4-diAP) on blueberry juice – induced relaxation of rat aortic rings ($n = 7$–8). $^{**}p < 0.01$ *vs.* no 3,4-diAP.

significant interaction between 3,4-diAP and BBJ [$F(2, 44) = 5.93$, $p = 0.006$]. Post hoc analysis indicated that this potent Kv blocker significantly blocked the relaxant effect of both the 1/100 and 1/50 dilutions of BBJ ($p < 0.001$). There was no significant impact of 3,4-diAP on the 1/10 dilution of BBJ.

3.4 The role of the CSE/H_2S pathway

The CSE inhibitor D,L-propargylglycine (PPG) was used to investigate the role of the CSE/H_2S pathway in mediating the relaxant effects of BBJ. An independent T test [$t(13) = 2.16$, $p = 0.60$] showed that there was no significant difference between the tension generated by noradrenaline alone (1.53 g ± 0.25 above baseline), and the tension generated by noradrenaline in the presence of PPG (1.37 g ± 0.52 above baseline). For

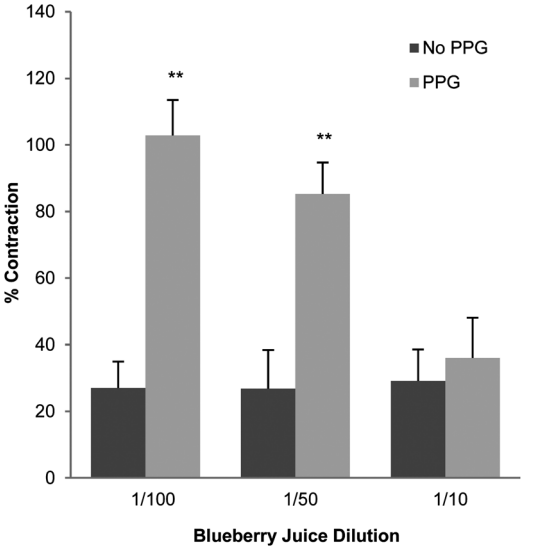

Fig. 6 The effect of propargylglycine (PPG) on blueberry juice – induced relaxation of rat aortic rings ($n = 7$–8). $^{**}p < 0.01$ *vs.* no PPG.

each aortic ring, the effect of BBJ in the presence or absence of PPG was calculated as a percentage of the NA-induced contraction in that tissue (Fig. 6). A two-way ANOVA demonstrated that there was a significant effect of PPG [$F(1, 44) = 34.05$, $p < 0.01$] and a significant interaction between PPG and BBJ [$F(2, 44) = 6.58$, $p = 0.03$]. PPG fully blocked the relaxant effect of 1/100 dilution of blueberry, and partially blocked the effect of the 1/50 dilution. Tukey HSD post hoc analysis revealed that the effect of PPG was highly significant at the 1/100 juice dilutions ($p < 0.001$) and at the 1/50 dilution ($p = 0.002$). PPG had no significant impact on the 1/10 dilution of BBJ.

4 Discussion

Elevated blood pressure is a prominent risk factor for cardiovascular disease and stroke. It has been estimated that a reduction of 3 mmHg in systolic blood pressure across a population would decrease mortality from stroke (by 8%) and coronary heart disease (by 5%).[34] In adults aged between 40 and 89, Lewington *et al.* identified a direct relationship between blood pressure and the risk of mortality from heart disease and stroke, even within the limits of normal blood pressure.[35] Therefore, maintenance of low blood pressure should be a high priority throughout life, and hence the identification and characterisation of dietary agents that may lower blood pressure is of huge importance. In addition, the use of dietary strategies in early stage hypertension or in prehypertension may delay the requirement for medication. Dietary strategies may also facilitate the use of lower dose drug regimes in people with hypertension.[17] For these reasons, investigation of the effects of dietary agents on healthy vascular tissue is of great interest.

Fruits and vegetables are well known to be high in antioxidants, and vegetarian diets have been associated with lower blood pressure.[36,37] A number of fruits and fruit juices have been studied in relation to their effects on blood pressure, and there is evidence that pomegranate,[38] cranberry,[22] and grape juice[39] have blood pressure-lowering effects. Blackcurrants have also been identified as having potent vasorelaxing properties.[25] In this study, two assays were utilised to compare the antioxidant status of BBJ with pomegranate juice, cranberry juice, grape juice and blackcurrant cordial. Using the Folin–Ciocalteu assay, the total polyphenol content of BBJ far exceeded that of any of the other beverages studied, with grape juice having the lowest total polyphenol content. A similar pattern was observed when the radical scavenging activity of the beverages was measured using the DPPH assay. BBJ had the highest mean radical scavenging activity, although in our study it only reached statistical significance in comparison to grape juice. The finding that BBJ has a high antioxidant capacity is in agreement with some other studies.[2–4] However, our conclusions are at variance with the findings of Seeram *et al.* who compared the antioxidant capacity of a number of juices, and found that pomegranate juice had a higher antioxidant capacity than blueberry juice.[40] Reasons for the different findings could include different countries of origin of the fruits, different processes used in the production of the juices, and different protocols used in the assays. Such differences between laboratories are very common, making it difficult to compare studies reporting antioxidant content of fruits, vegetables and juices.[41] Despite these differences however, the results of this study and others indicate that blueberry juice has a high antioxidant capacity, suggesting that it may be useful for the treatment or prevention of any condition involving inflammation and oxidative stress. In relation to vascular function, there is evidence that oxidative stress occurs at the vascular level in hypertension,[42] and that inhibition of reactive oxygen species can lower blood pressure.[43]

The hypothesis that BBJ may have vasorelaxing properties is based on the results of studies in humans with obesity and metabolic syndrome[14,15] as well as some studies in rats.[5,9,10,13,16] Similar to observations in the dietary studies by Norton *et al.*[13] and Kalea *et al.*,[16] we observed that when BBJ was added *in vitro* to the tissue bath prior to the addition of noradrenaline, BBJ potently blocked the NA-induced contractions. This was significant at dilutions of juice equal to and less than 1/250. The vasorelaxing effect appeared to reach a maximum at a dilution of 1/100. At this dilution the mean NA-induced contraction was only 27% of the NA-induced contraction in the absence of BBJ. There was no further relaxing effect at dilutions of 1/100, 1/50 or 1/10. These dilutions of BBJ were then used to investigate the mechanism of action of the juice.

Overall, this study clearly shows that blueberry juice has potent vasorelaxing properties *in vitro*. As described above, it also provides some *in vitro* evidence to indicate that blueberry juice may have a higher antioxidant capability than some other commercially available fruit beverages. However, conclusions from any *in vivo* study must of course be interpreted alongside an awareness of bioavailability issues *in vivo*. The bioavailability of fruit polyphenols is generally believed to be low.[44] However, as reviewed by Prior and Wu,[45] and more recently by del Rio *et al.*,[46] whilst a number of studies have investigated the absorptive and metabolic profiles of various polyphenol constituents of blueberries, ascertaining the biological availability of ingested fruits, vegetables and juices is a complicated process. Blueberries contain more than 14 different anthocyanins, in addition to other polyphenols.[45] Extensive metabolism occurs,[45,47] producing metabolites, at least some of which may also have bioactivity. As explained by Del Rio *et al.*,[46] this large array of different bioactive constituents, some of which may individually be present at low levels, makes it difficult to measure levels in biological fluids. Also, the patterns of metabolism are often complicated, and it can be difficult to clearly identify some of the metabolites. Therefore, it may be some time before an accurate pharmacokinetic profile of all of the constituents of dietary blueberries and blueberry juice can be fully elucidated. Notwithstanding these issues however, there is a growing body of evidence demonstrating that blueberries taken in the diet have beneficial effects on cardiovascular[14] and endocrine functions[15] in humans, as well as on markers of oxidative stress and inflammation.[48,49] The results of these studies indicate that blueberry consumed in the diet does indeed have biological activity *in vivo.* Many feeding studies in animals have also been performed demonstrating effects of blueberry on cardiovascular function,[5–7,10,13,16,50] but more human studies will ultimately be necessary to fully establish the

in vivo effects of dietary consumption of blueberry juice on human vascular function and blood pressure.

Control of blood pressure is a complex process, regulated by many central and peripheral mechanisms. Within the vasculature, there is an abundance of signaling pathways and ion channels that play a role in controlling contractility of the blood vessels. This study undertook to perform an *in vitro* investigation of two factors that are known to be key players in the regulation of the vasculature: the voltage-gated potassium channels (Kv) and the hydrogen sulphide (H_2S) pathway. The physiological roles of potassium channels in vascular smooth muscle was reviewed by Nelson and Quayle in 1995 [51] and by Ko *et al.* in 2008.[26] Opening of potassium channels in the smooth muscle membrane leads to efflux of potassium, which causes hyperpolarisation and subsequent vasodilation. There is also evidence that opening potassium channels leads to nitric oxide-mediated relaxation of large mesenteric arteries in the rat.[52] Conversely, inhibition of K^+ channels causes vasoconstriction. Many pharmacological vasodilators exert their antihypertensive affects by this mechanism. It is known that many common agents, such as β-adrenergic agonists and adenosine exert their receptor-mediated effects by opening K^+ channels in the vascular cell membrane, *via* second messenger systems. Also some vasodilators, such as pinocidil and the recently developed iptakalim, act directly on potassium channels.[53,54] In this study, Kv channels were investigated as they are known to be critical players in the maintenance of vascular tone, and some types have been shown to be dysfunctional in models of hypertension.[55–57] For this purpose, the non-selective Kv channel blockers 4-AP and 3,4-diAP were utilised. Neither drug was found to have any significant effect on NA-induced contractions in their own right. However, both of them interacted with the BBJ to cause significant blockade of the vasorelaxing effect of the juice. 4-AP significantly blocked the effect of the 1/100 dilution of BBJ, partially (80.5%) restoring the NA-induced contraction. This Kv blocker appeared to block the effect of the 1/50 dilution of BBJ to some extent but this was not significant, and the 1 mM 4-AP had no impact on the powerful vasorelaxation caused by 1/10 BBJ. A similar effect was seen with 3,4-diAP, although it was more potent than 4-AP at blocking the effect of the juice. 3,4-diAP (1 mM) powerfully blocked the effect of both 1/100 and 1/50 dilutions, restoring the NA-induced contractions almost fully (97% and 93% respectively). This observation is in line with the literature, as 3,4-diAP has been reported to be 50 times more potent than 4-AP.[58] Similar studies have demonstrated that vasorelaxation caused by apple procyanidins can be blocked by 4-AP as well as by other K^+ channel blockers.[23,24] It appears that these fruit-derived polyphenols may act as relaxing factors by activating Kv (and possibly other K^+) channels and causing hyperpolarisation. The full extent of the signalling mediators involved in this mechanism requires further study however.

H_2S has been identified as a gasotransmitter critical to many physiological processes, similar to nitric oxide and carbon monoxide.[59] In the vascular system, H_2S is generated from L-cysteine by the enzyme cystathionine γ-lysase (CSE). It has a vasorelaxant effect on arteries, and this has been attributed mainly to the opening of K_{ATP} channels as a result of *S*-sulfhydration of specific cysteine residues of the channel protein.[29] There is also some evidence that H_2S may act as a Kv channel opener in arterial walls.[30,31] In addition, H_2S has other targets that may also play roles in mediating vasorelaxation.[60] It was first shown by Zhong *et al.*[61] and also by Yan *et al.*[62] that this pathway plays a crucial role in the maintenance of normal blood pressure. A number of other studies have similarly observed that blood pressure is increased by the inhibition or knock-out of CSE,[63] and reduced by administration of NaHS.[64] Hence the vasorelaxing effects of H_2S make this pathway an ideal target for the treatment of hypertension.[65] Thus given the body of evidence to support a role for H_2S in regulating vascular tone, the CSE inhibitor propargylglycine (PPG) was used to investigate the interaction of BBJ with the H_2S pathway. PPG had no significant effect on NA-induced contraction in its own right. However, similar to 3,4-diAP, it potently blocked the vasorelaxant effect of BBJ. It fully blocked the effect of 1/100 dilution of BBJ. It also had a powerful blocking effect (85%) against the 1/50 dilution of BBJ. This study demonstrated that inhibition of the CSE enzyme blocked the effects of BBJ, providing very strong evidence that H_2S is produced in response to BBJ. As with the Kv blockers, the extent of that blockade was dependent on the concentration of BBJ present. Given the effects of the Kv blockers, it is possible that BBJ polyphenols cause the production of H_2S, which then mediates vasorelaxation by opening Kv (and possibly other K^+) channels. Indeed it is also possible that the juice polyphenols, as well as increasing the availability of H_2S, also cause activation of the Kv channels independently of H_2S. Further studies are required to further investigate the mechanism.

5 Conclusion

This study presents some novel findings that are of interest to the study of vascular physiology and hypertension, as well as to society in general. Firstly, the observation that BBJ has higher antioxidant capacity than some other very well-reputed juices is of interest. Furthermore, BBJ potently inhibits NA-induced contraction *in vitro*, suggesting that as a dietary constituent, it may be useful for the prevention and treatment of hypertension. In addition, the study presents novel evidence to indicate that Kv channels may be central to mediating the actions of BBJ. Of major interest is the observation that inhibition of the CSE/H_2S pathway could potentially abolish the effect of BBJ, indicating that BBJ may be mediating its vasorelaxing effects *via* the activation of this pathway. This mechanism of action has not hitherto been explored in relation to blueberry juice. Further studies are required however to elucidate the full mechanisms involved. In addition, whilst the beneficial effects of berries and other fruit are generally attributed to their antioxidant capabilities, the link between antioxidant activity and the mechanistic pathways described in this study are not clear. Future studies will be needed to further explore blueberry juice and to fully unravel the complexities involved.

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