Resveratrol shows neuronal and vascular-protective effects in older, obese, streptozotocin-induced diabetic rats

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Abstract

Diabetes-induced CVD is the most significant complication of prolonged hyperglycaemia. The aim of this study was to determine whether resveratrol, a polyphenol antioxidant compound, when administered at a dose that can be reasonably obtained through supplementation could prevent the development of cardiovascular complications in older, obese, diabetic rats. Diabetes was induced in 6-month old, obese, male Wistar rats via a single intravenous dose of streptozotocin (65 mg/kg). Randomly selected animals were administered resveratrol (2 mg/kg) via oral gavage daily for 8 weeks. Body weights, blood glucose levels, food intake and water consumption were monitored, and assessments of vascular reactivity, tactile allodynia and left ventricular function were performed. Resveratrol therapy significantly improved tactile allodynia and vascular contractile functionality in diabetic rats ($P < 0.05$). There were no significant changes in standardised vasorelaxation responses, plasma glucose concentrations, water consumption, body weight, left ventricular hypertrophy, kidney hypertrophy, heart rate or left ventricular compliance with resveratrol administration. Resveratrol-mediated improvements in vascular and nerve function in old, obese, diabetic rats were associated with its reported antioxidant effects. Resveratrol did not improve cardiac function nor mitigate the classic clinical symptoms of diabetes mellitus (i.e. hyperglycaemia, polydypsia and a failure to thrive). This suggests that supplementation with resveratrol at a dose achievable with commercially available supplements would not produce significant cardioprotective effects in people with diabetes mellitus.

Key words: Resveratrol: Type 1 diabetes: Age: Obesity: Streptozotocin rats

Diabetes mellitus (DM) is a chronic, metabolic disease in which insufficient or absent secretion of insulin impairs glucose uptake from the bloodstream causing hyperglycaemia[1–4]. Hyperglycaemia promotes oxidative stress via overproduction of reactive oxygen species (ROS) inducing lipid peroxidation, mitochondrial dysfunction, altered signal transduction, abnormal gene expression and cell apoptosis[1,5–10]. In turn, these chemical and metabolic changes manifest many of the severe complications common to individuals with DM, including renal failure, CVD, endothelial dysfunction, hyperalgesic pain (diabetic neuropathy) and blindness[6,11–13]. Diabetes-induced CVD is the most significant complication of prolonged hyperglycaemia and is estimated to be responsible for approximately 80 % of deaths in people with DM[12,14,15].

Considering the substantial role of oxidative stress in the aetiology of DM, nutritional antioxidant therapies have considerable potential as preventative agents against the development of secondary complications associated with this condition[16–19]. The polyphenolic phytoalexin resveratrol (3,5,4-trihydroxystilbene) is one such antioxidant compound. Resveratrol is found naturally in a variety of foods including grape skins, blueberries, cranberries, *Polygonum cuspidatum* (Japanese knot-weed), peanuts and red wines[20–23].

Numerous studies have reported the benefits of resveratrol administration in the treatment of various CVD including coronary artery disease, cardiac arrhythmias, myocardial infarction, heart failure and cardiac fibrosis[20–24]. The cardioprotective effects of resveratrol are mostly attributable to its antioxidant properties, particularly its free-radical scavenging capacity and ability to up-regulate the expression of endogenous antioxidant genes[25–27]. Resveratrol has also demonstrated anti-inflammatory, anti-cancer, anti-ageing, anti-viral and anti-platelet effects[17,21,23,28–34].

The health benefits of resveratrol, however, appear to be dose dependent. A prospective cohort study on 1270 Tuscans over the age of 65 years found that dietary resveratrol levels were not associated with CVD, inflammatory markers, cancer or a lower all-cause mortality risk[35]. Conversely, studies prescribing

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resveratrol supplements have reported improvements in oxidative stress, systolic blood pressure (SBP) and blood glucose and cholesterol levels in both healthy individuals and patients with DM[36–38]. Accordingly, these findings indicate that supplementation with resveratrol may improve cardiovascular and oxidative stress outcomes, particularly in individuals with DM.

The streptozotocin (STZ) rat is one of the most utilised animal models in diabetes research with well over 18 000 PubMed citations (counted in 2016). Typically, young adult rats (8–12 weeks of age when diabetes is induced) are used to investigate the complications of DM and its treatment[34,39,40]. Independently, older Wistar rats are known to develop the metabolic syndrome/insulin resistance with age as visceral and retroperitoneal fat mass increases[41,42]. Although the cardioprotective effects of resveratrol in younger STZ-induced diabetic rats have been investigated, there are limited data on its effectiveness in older, obese rats – a model that may be more representative of type 1 DM with some aspects of type 2 DM[43]. Accordingly, this study aimed to assess whether resveratrol, when administered at a dose that can be reasonably achieved using commercially available supplements, could prevent the development of diabetes-induced cardiovascular complications in old, obese, STZ-induced diabetic rats.

Methods

Animal models and treatment regimen

In all, thirty male Wistar (WIS) rats (obtained from the Animal Resource Centre, Perth, WA) were randomised to one of the following four treatment groups: control (WIS, *n* 8), control treated with resveratrol (WIS + Res, *n* 8), STZ-induced diabetic model (STZ, *n* 7) and STZ-diabetic model treated with resveratrol (STZ + Res, *n* 7). Treatments for all animals began once they had reached 6 months of age and had become obese (i.e. >500 g = body mass consistent with Wistar fatty rats)[44]. Diabetes was induced by a single injection of STZ (65 mg/kg) into the femoral vein. Water consumption and body weights of all rats were assessed weekly. Animals that were hyperglycaemic (>15 mmol/l) and exhibited polydipsia and a failure to thrive (i.e. weight loss) were considered to be diabetic. Insulin was not administered to any of the rats during the study. Resveratrol was delivered as a bolus dose of 2 mg/kg via oral gavage daily for 8 weeks. This dose was chosen as it can be easily achieved in humans using commercially available resveratrol supplements and has been used successfully in previous investigations with young rats[37,38,45–47]. Animals were housed in a constant 12 h light–12 h dark cycle at a temperature of 22 (SEM 2)°C and were allowed access to water and food *ad libitum*. All experimental procedures involving animals were approved by the Animal Ethics Committee of Central Queensland University under guidelines from the National Medical Research Council of Australia.

Assessment of neuropathic pain

Tactile allodynia was assessed at the end of the treatment period using von Frey filaments. Filaments were applied to the plantar surface of the animal's hind paw in increasing tensile strength (3 through to 20 g) until the chosen filament flexed or a brisk paw-withdrawal response was observed[39]. Only light touches were applied to prevent tissue damage, and if there was no response 20 g was recorded.

Blood collection and haemodynamic and biochemical assessment

Before euthanasia, animals were sedated and their heart rate (HR) and SBP were measured using tail cuff plethysmography (ADInstruments). HR and SBP measurements were performed in the same environment animals were housed in (temperature 22 (SEM 2)°C) with three recordings taken per measurement. Familiarisation sessions were run before collection of data to ensure that representative responses were measured. All rats were then euthanised via an intraperitoneal injection of sodium phenobarbitone (100 mg/kg) and heparinised (1000 U/100 g) intravenously into the right femoral vein. Blood samples were collected from the abdominal vena cava allowing for the assessment of plasma glucose levels using Precision Plus Blood Glucose Electrodes (Medisense, Abbott Laboratories). Wet mass measurements of the left ventricle, right ventricle and kidneys, as normalised to body mass, were also recorded.

Assessment of vascular function

Assessment of vascular reactivity was performed according to Vella *et al.*[46]. Thoracic aortas with intact endothelium were carefully isolated and suspended within 25 ml organ baths containing gassed (O_2 (95 %)/CO_2 (5 %)) Tyrode's solution (all in mM concentrations: NaCl 136·9, KCl 5·4, $MgCl_2$ 1·05, NaH_2PO_4 0·42, $NaHCO_3$ 22·6, $CaCl_2$ 1·8, glucose 5·5, ascorbic acid 0·28, EDTA 0·1; pH approximately 7·4). The aortic rings were set at a resting tension of 10 mN and were allowed to equilibrate for 30 min before being exposed to cumulative concentrations of noradrenaline, acetylcholine and sodium nitroprusside. Dose–response curves to acetylcholine and sodium nitroprusside were completed in the presence of a submaximal contraction to noradrenaline (3×10^{-6} M concentration). Any fluctuations in tension were measured using transducers and recorded using Chart software and PowerLab® data acquisition units.

Assessment of left ventricular function

Langendorff heart preparations were used to measure left ventricular function[46]. In short, hearts were isolated and perfused retrogradely via an aortic cannula with gassed (O_2 (95 %)/CO_2 (5 %)) modified Krebs–Henseleit buffer (all in mM concentrations: NaCl 119·1, KCl 4·75, $MgSO_4$ 1·19, KH_2PO_4 1·19, $NaHCO_3$ 25·0, glucose 11·0 and $CaCl_2$ 2·16; pH approximately 7·4) and maintained at 37°C with a constant pressure of 100 mmHg. Isovolumic contractile left ventricular function was assessed by inserting a latex balloon catheter connected to a pressure transducer into the left ventricle. The balloon volume was adjusted to 0 mmHg of diastolic pressure and hearts were paced at 250 beats/min using an artificial stimulation source. End-diastolic pressure was

recorded for 3 min at 5 mmHg increments over a range of 0–30 mmHg to allow for calculation of diastolic stiffness and left ventricular developed pressure. Contractile function was measured by calculating maximal and minimal $+dP/dt$ values together at a diastolic pressure of 10 mmHg.

Drugs and chemicals

Resveratrol, STZ, noradrenaline, acetylcholine and sodium nitroprusside were purchased from the Sigma Chemical Company. All serial dilutions of noradrenaline, acetylcholine and sodium nitroprusside were made using distilled water. Reagents and chemicals utilised in the preparation of buffers were of analytical grade and purchased from the Sigma Chemical Company and Thermo Fisher Scientific.

Statistical analysis

All data are presented as mean values with their standard errors. The results were analysed using Student's independent samples t test and a two-way ANOVA with Bonferroni *post hoc* tests where appropriate. All statistical analyses were performed using Graph-Pad Prism software version 3.0 for Windows (GraphPad Software, Inc.). Statistical significance was set at an α level of $P < 0.05$.

Results

Biometric parameters

Significant increases in blood glucose and water intake, in conjunction with an impaired ability to gain weight, confirmed that diabetes had been successfully induced in the experimental rats (Table 1, Fig. 1 and 2). Administration of resveratrol had no significant effect on blood glucose concentration with levels remaining 2·8 times higher in the STZ + Res rats *v.* the control group (Table 1). STZ + Res rats showed a significant increase in water consumption and decrease in body mass compared with the STZ animals from day 14 to 35 of the treatment period (Fig. 1 and 2). There were no differences in water intake and body mass between the STZ and the STZ + Res groups by the end of the study period (days 42–56) (Fig. 1 and 2).
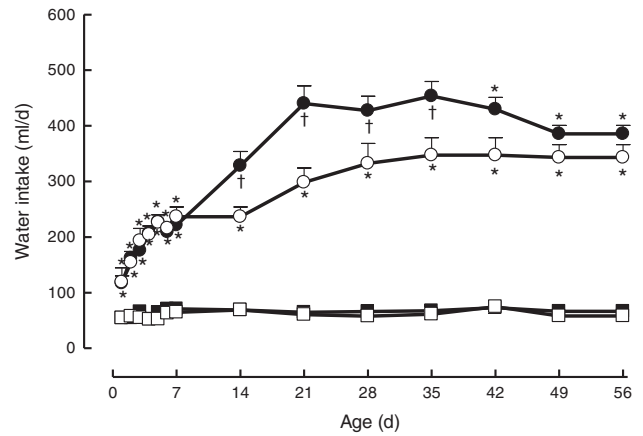
Fig. 1. Weekly water consumption for control (WIS (–□–, *n* 8), control + resveratrol (–■–, *n* 8), streptozotocin (STZ (–○–, *n* 7) and STZ + resveratrol (STZ + Res (–●–, *n* 7) groups. Values are means, with their standard errors. * $P < 0.05$ *v.* WIS, † $P < 0.05$ *v.* STZ. WIS, Wistar.
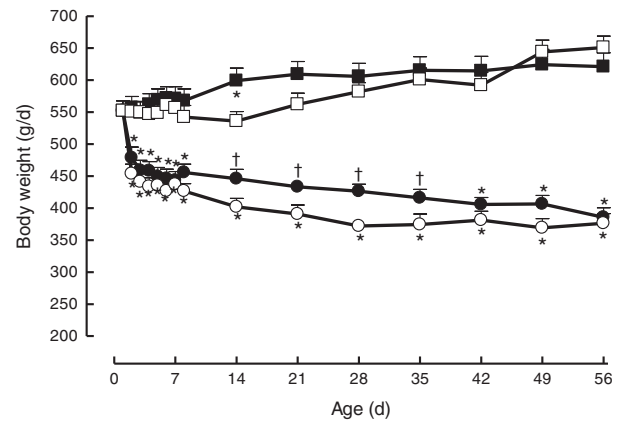
Fig. 2. Weekly body weight for control (WIS (–□–, *n* 8), control + resveratrol (WIS + Res (–■–, *n* 8), streptozotocin (STZ (–○–, *n* 7) and STZ + resveratrol (STZ + Res (–●–, *n* 7) groups. Values are means, with their standard errors. * $P < 0.05$ *v.* WIS, † $P < 0.05$ *v.* STZ. WIS, Wistar.

Table 1. Physiological and biochemical parameters following resveratrol (Res) administration to control (WIS + Res) and diabetic (STZ + Res) rats
(Mean values with their standard errors)

Parameters	WIS (*n* 8) Mean	SEM	WIS + Res (*n* 8) Mean	SEM	STZ (*n* 7) Mean	SEM	STZ + Res (*n* 7) Mean	SEM
Plasma glucose (mmol/l)	10·93	1·04	11·33	1·43	30·60*	2·02	31·10*	2·82
Left ventricular mass (mg/g body mass)	1·66	0·07	1·90	0·09	2·15*	0·10	2·36*	0·09
Right ventricular mass (mg/g body mass)	0·37	0·03	0·44	0·04	0·48	0·04	0·52	0·03
Kidney mass (mg/gram body mass)	2·52	0·09	2·66	0·07	5·01*	0·16	5·20*	0·19
Systolic blood pressure (mmHg)	122·70	6·71	129·63	5·35	123·80	3·41	129·63	5·35
Developed pressure (mmHg)	113·00	7·31	96·00	6·52	63·83*	8·15	81·00*	2·77
Diastolic stiffness (K dimensionless)	22·51	0·71	20·71	1·76	15·38*	0·84	22·29†	0·87
$+dP/dT_{max}$ (mmHg/s)	2170·20	155·39	1887·60	69·42	1237·17*	158·68	1425·00*	57·06
$-dP/dT_{max}$ (mmHg/s)	−1358·80	131·90	−1121·80	52·06	−672·00*	110·45	−833·20*	43·11
Heart rate (beats/min)	448·50	26·18	479·25	11·98	363·20*	14·88	380·0*	10·7
Tactile allodynia (g)	17·10	0·69	18·63	0·71	6·33*	0·71	10·17†	0·31

WIS, Wistar; STZ, streptozotocin.
* $P < 0.05$ *v.* WIS, † $P < 0.05$ *v.* STZ.

Neuropathic pain

Diabetic rats developed significant tactile allodynia with the response threshold occurring at 6 g of force compared with 17 g in the control rats (Table 1). Resveratrol therapy significantly improved the development of tactile allodynia, increasing the withdrawal threshold to 10 g (Table 1).

Haemodynamic assessment

HR was significantly lower in the diabetic rats compared with the control animals and was not altered by resveratrol treatment (Table 1). SBP was not significantly different among any of the treatment groups. DM led to left ventricular and kidney hypertrophy, and this was not prevented by resveratrol (Table 1). Right ventricular mass was similar for all groups (Table 1).

Vascular reactivity in isolated thoracic aortic rings

Induction of DM and the administration of resveratrol increased vascular responsiveness to noradrenergic-mediated contractions (Fig. 3(a)). Diabetic rats also had impaired endothelial-dependent relaxation, an observation that was improved by resveratrol administration (Fig. 3(b)). Resveratrol therapy significantly enhanced endothelium-independent relaxation for both the STZ + Res and the WIS + Res groups (Fig. 3(c)). There were no significant differences in $\log_{10}EC_{50}$ and hillslope values for noradrenaline, acetylcholine and sodium nitroprusside (Table 2). Endothelium-independent relaxation normalised to percentage inhibition of the contraction induced by noradrenaline showed reduced vascular responsiveness in diabetic animals, and this was not reversed by resveratrol (Fig. 4(a)). Endothelium-independent relaxation responses normalised to noradrenaline contraction demonstrated no significant differences among the treatment groups (Fig. 4(b)).

Ex vivo left ventricular function

Diastolic stiffness was significantly reduced in the untreated diabetic rats compared with the control groups but was normalised by resveratrol therapy (Table 1). Ventricular contractility ($+ dP/dt$), relaxation ($-dP/dt$) and developed pressure
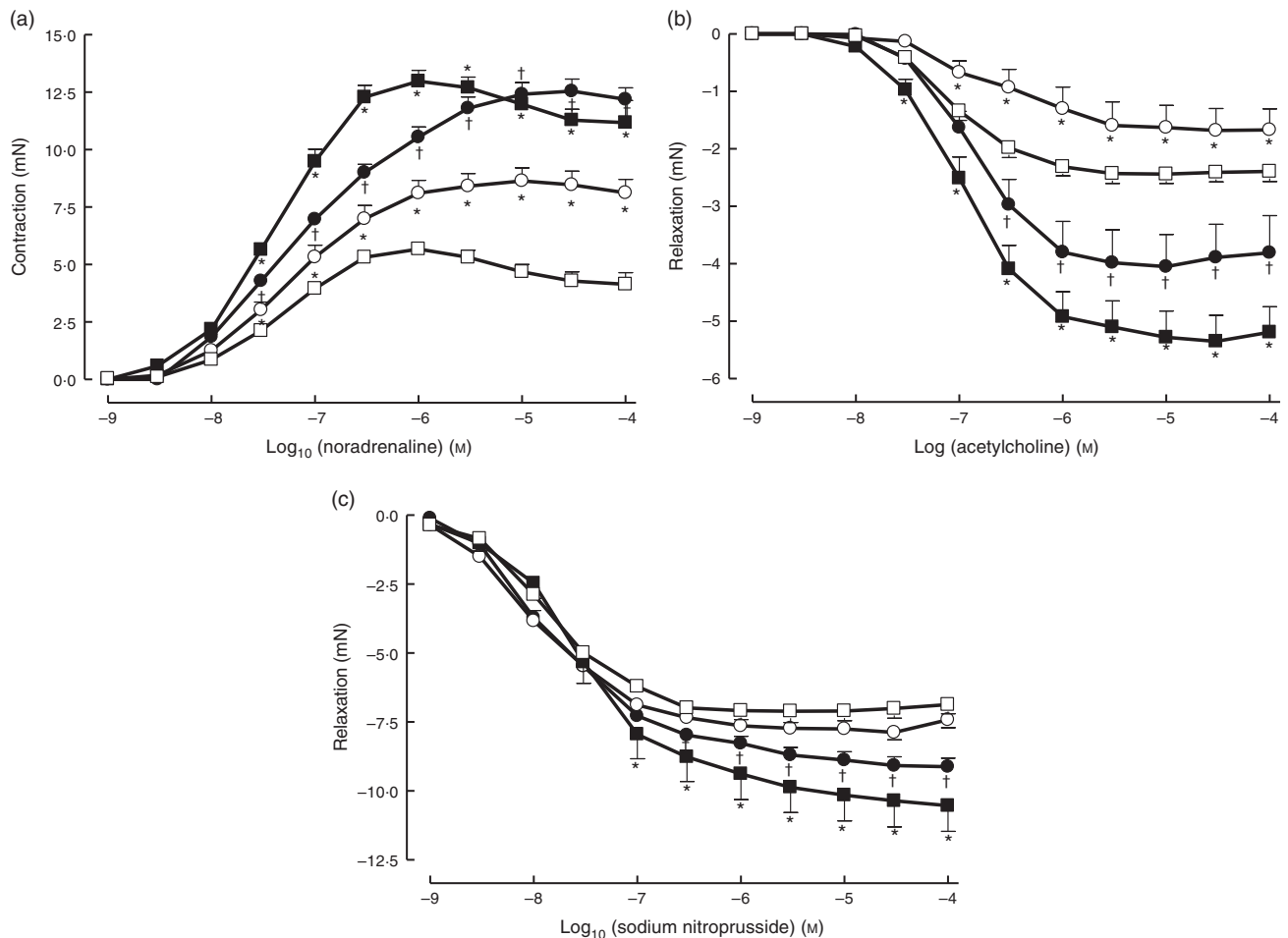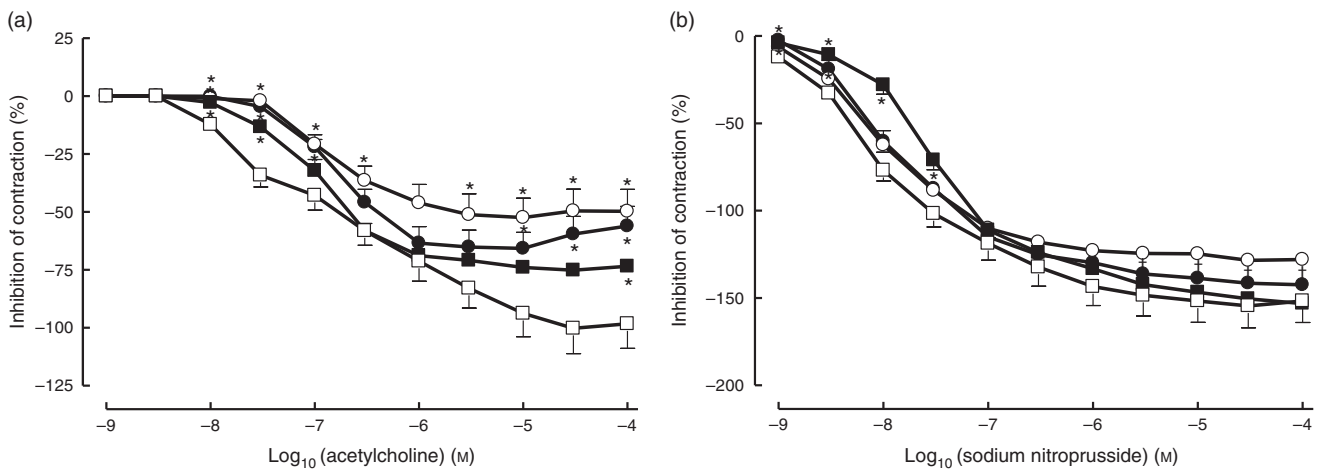

Fig. 3. (a) Cumulative concentration response to noradrenaline in isolated thoracic aortic rings from control (WIS (—□—, *n* 8), control + resveratrol (WIS + Res (—■—, *n* 8), streptozotocin (STZ (—○—, *n* 7) and STZ + resveratrol (STZ + Res (—●—, *n* 7) groups. Values are means, with their standard errors. * $P < 0.05$ *v.* WIS, † $P < 0.05$ *v.* STZ. (b) Cumulative concentration response to acetylcholine in isolated noradrenaline pre-contracted thoracic aortic rings from WIS (*n* 8), WIS + Res (*n* 8), STZ (*n* 7) and STZ + Res (*n* 7) groups. (c) Cumulative concentration response to sodium nitroprusside in isolated noradrenaline pre-contracted thoracic aortic rings from WIS (*n* 8), WIS + Res (*n* 8), STZ (*n* 7) and STZ + Res (*n* 7) groups. WIS, Wistar.

Table 2. $Log_{10}EC_{50}$ and hillslope values in isolated thoracic aortic rings following resveratrol (Res) administration to control (WIS + Res) and diabetic (STZ + Res) rats
(Mean values with their standard errors)

Parameters	WIS (n 8)		WIS + Res (n 8)		STZ (n 7)		STZ + Res (n 7)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Noradrenaline ($log_{10}EC_{50}$)	−7·326	0·1406	−7·411	0·09197	−7·211	0·1474	−7·072	0·08836
Acetylcholine ($log_{10}EC_{50}$)	−6·7870	0·34290	−6·905	0·2077	−6·840	0·2335	−6·858	0·2598
Sodium nitroprusside ($log_{10}EC_{50}$)	−7·682	0·2355	−7·430	0·2426	−7·827	0·07111	−7·681	0·07779
Noradrenaline (hillslope)	1·212	0·4123	1·181	0·2581	0·9684	0·2793	0·7912	0·1126
Acetylcholine (hillslope)	−0·6771	0·3427	−1·234	0·6532	−1·417	0·9694	−1·333	0·9545
Sodium nitroprusside (hillslope)	−0·7691	0·2934	−0·8882	0·3930	−0·9764	0·1412	−0·8699	0·1223

WIS, Wistar; STZ, streptozotocin.

Fig. 4. (a) Cumulative concentration response to acetylcholine as a percentage inhibition of the contraction induced by noradrenaline in isolated noradrenaline pre-contracted thoracic aortic rings from control (WIS (—□—, n 8), control + resveratrol (WIS + Res (—■—, n 8), streptozotocin (STZ (—○—, n 7) and STZ + resveratrol (STZ + Res (—●—, n 7) groups. Values are means, with their standard errors represented by vertical bars. * $P < 0.05$ v. WIS. (b) Cumulative concentration response to sodium nitroprusside as a percentage inhibition of the contraction induced by noradrenaline in isolated noradrenaline pre-contracted thoracic aortic rings normalised to from WIS (n 8), WIS + Res (n 8), STZ (n 7) and STZ + Res (n 7) groups. Values are means, with their standard errors. * $P < 0.05$ v. WIS, † $P < 0.05$ v. STZ. WIS, Wistar.

were substantially decreased in the diabetic animals, and this was not significantly altered by resveratrol therapy (Table 1).

Discussion

This study aimed to assess whether resveratrol administration to diabetic animals, at a dose that can be reasonably achieved with supplementation, could produce improvements in cardiovascular function.

Resveratrol supplementation in old diabetic animals successfully inhibited increased nerve sensitivity. This attenuation of diabetic neuropathy likely reflects the ROS scavenging capabilities of resveratrol, preventing neuronal cell death, as reported previously[48–50].

STZ-induced DM is known to increase oxidative stress and subsequently impair endothelial and smooth muscle function in isolated thoracic aortas[51–53]. Consistent with these findings, we observed significantly reduced endothelium-dependent and endothelium-independent relaxation in thoracic aortic rings isolated from STZ rats. Supplementation with resveratrol substantially improved vasorelaxation and increased maximal contractile response of the blood vessels. These results reflect

previous reports that resveratrol can improve vaso-relaxant and vaso-contractile capacity as a result of its ability to reduce free radical production[16,19,46,52–55].

Relaxation responses normalised to percentage inhibition of the contraction induced by noradrenaline also showed impaired endothelial function in the STZ rats; however, there was no effect of resveratrol administration. There were also no 1differences overall between any of the groups when endothelium-independent relaxation responses were standardised to inhibition of noradrenaline contraction. Similarly, $log_{10}EC_{50}$s and hillslope values for noradrenaline, acetylcholine or sodium nitroprusside were similar for all treatment groups. Ultimately, these findings suggest that the differences observed in maximal contraction and relaxation responses in STZ- and/or resveratrol-treated animals were not due to differences in the pharmacological response of each tissue but due to changes in the physiology of the vasculature itself.

Data from human clinical trials examining the effectiveness of resveratrol in attenuating diabetes-induced CVD indicate that the positive outcomes of resveratrol administration seen in animal studies are not entirely translatable to humans. An investigation into the effects of short-term (4 week) resveratrol

(1·5 g/d) treatment in obese, mildly insulin-resistant men found that resveratrol had no significant effect on SBP and diastolic blood pressure, inflammatory biomarkers, insulin sensitivity or HbA1c levels[56]. A non-significant effect of resveratrol on SBP in type 2 diabetic patients has also been reported[57]. A recent meta-analysis concluded that resveratrol produced small but statistically significant improvements in SBP, creatinine and HbA1c in subjects with type 2 DM but, on average, had no effect on fasting glucose, insulin, diastolic blood pressure, cholesterol or TAG levels[58].

Similar to these findings, we observed that supplementation with resveratrol was unable to lower blood glucose levels, prevent left ventricular remodelling or improve left ventricular pump function in old, obese, diabetic rats. These results are in contrast to other studies that have found that resveratrol prevented left ventricular hypertrophy and normalised left ventricular compliance in 8-week-old diabetic, deoxycorticosterone acetate (DOCA)-salt and spontaneously hypertensive rats[46,52,59]. Likewise, administering resveratrol to 8–10-week-old diabetic rats has been reported to decrease plasma glucose levels by approximately 20 %[34,60].

The discrepancy between these results and those of the present study may be due to the different age groups of the rats used in each experiment (i.e. 8–10-week *v.* 6-month-old rats). Ageing in Wistar rats has been associated with insulin resistance, meaning that the older rats used in this study could have negatively influenced the cardiac response and hypoglycaemic effects of resveratrol[41,42]. Nevertheless, in a previous study using 8-week-old rats, we also observed no significant effect of resveratrol on plasma glucose levels when administered at the same dose of 2 mg/kg per d for 8 weeks[46]. Considering that the other investigations administered resveratrol at doses of 0·5 mg/kg thrice daily for 2 weeks and 0·75 mg/kg thrice daily for 8 weeks, it is possible that the frequency of dosing (i.e. one *v.* multiple doses per d) could have influenced resveratrol's glucose-lowering properties[34]. Older animals also show negative age-related alterations in cardiovascular function and poorer ROS scavenging capacity[61,62]. In this study, these changes in antioxidant ability could have posed an additional pathophysiological challenge for resveratrol to overcome.

The findings of the present investigation match those of human studies that indicate that the animal model used here (i.e. 6-month old, obese, diabetic rats) may be a more accurate representation of the ageing human patient with type 1 or type 2 DM. These similarities also mean that the lack of improvement in cardiac function and glucose levels observed in the STZ + Res animals was not so unexpected. Ultimately, there is insufficient evidence to fully support the viability of resveratrol as a nutritional supplement to improve metabolic health[56–66].

Conclusion

Although resveratrol was successful in preserving vasocontractile and nerve functionality in this study, in reality, supplementation with resveratrol at a dose achievable with commercially available supplements may not be a viable all-round treatment for DM. The cardioprotective effects of resveratrol observed in this investigation were not as extensive as those reported by previous studies and may be associated with the age and degree of obesity in these rats. The similarity between the findings of the present investigation and human studies indicates that the animal model used in these experiments (i.e. 6-month old, obese, diabetic rats) may be a more accurate representation of type 1 DM, and aspects of type 2 DM, in older humans.

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