British Journal of Nutrition (2009), **101**, 1165–1170
doi:10.1017/S0007114508073637

The antioxidant effects of quercetin metabolites on the prevention of high glucose-induced apoptosis of human umbilical vein endothelial cells

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(Received 7 April 2008 – Revised 17 July 2008 – Accepted 25 July 2008 – First published online 14 November 2008)

Diabetes mellitus is an important risk factor for CVD. A previous study showed that high glucose induced the apoptosis of human umbilical vein endothelial cells (HUVEC) via the sequential activation of reactive oxygen species, Jun N-terminal kinase (JNK) and caspase-3. The apoptosis cascade could be blocked by ascorbic acid at the micromolar concentration (100 μM). In addition to ascorbic acid, quercetin, the most abundant dietary flavonol, has been recently actively studied in vascular protection effects due to its antioxidant effect at low micromolar concentrations (10–50 μM). Quercetin sulfate/glucuronide, the metabolite of quercetin in blood, however, has been rarely evaluated. In the present study, we investigated the effect of quercetin sulfate/glucuronide on the prevention of high glucose-induced apoptosis of HUVEC. HUVEC were treated with media containing high glucose (33 mM) in the presence or absence of ascorbic acid (100 μM) or quercetin sulfate/glucuronide (100 nM, 300 nM and 1 μM). For the detection of apoptosis, a cell death detection ELISA assay was used. The level of intracellular H_2O_2 was measured by flow cytometry. JNK and caspase-3 were evaluated by a kinase activity assay and Western blot analysis. The results showed that high glucose-induced apoptosis was inhibited by quercetin sulfate/glucuronide in a dose-dependent manner. The effect of quercetin sulfate/glucuronide on H_2O_2 quenching, inhibition of JNK and caspase-3 activity at the nanomolar concentration (300 nM) was similar to that of ascorbic acid at the micromolar concentration (100 μM). The findings of the present study may shed light on the pharmacological application of quercetin in CVD.

Quercetin metabolites: Glucose: Endothelial cells: Apoptosis: Reactive oxygen species: Jun N-terminal kinase: Caspase

Diabetes mellitus is a major risk factor of cardiovascular atherosclerosis[1]. High glucose-induced endothelial cell apoptosis[2–5], which may disturb the integrity of the endothelial monolayer, plays a pivotal role in diabetes-associated vascular dysfunction, including atherosclerosis[6]. In cultured human umbilical vein endothelial cells (HUVEC), high glucose has been found to generate H_2O_2, which in turn activates Jun N-terminal kinase (JNK), triggers caspase-3 and finally leads to apoptosis. The high glucose-induced H_2O_2 generation, JNK activation, triggering of caspase-3 and facilitation of apoptosis could be suppressed by ascorbic acid[2]. To reduce the cardiovascular events, ascorbic acid, one of the most common antioxidants, has been widely used to improve vascular function[7,8]. However, the results of clinical studies have been disappointing[9–11]. The discrepancy between the experimental and clinical studies may be because the concentrations of ascorbic acid used in experiments were much higher than those detected in humans[12,13].

To seek other strong antioxidants, interest has grown in dietary flavonoids, which are mainly derived from vegetables and fruits. Flavonoids represent the major class of polyphenolics, and comprise several thousand plant-derived compounds with a common skeleton of phenylchromane[14]. Several epidemiological studies have shown an inverse correlation between the dietary consumption of flavonols and flavones (two main classes of flavanoids) and mortality from CVD[15,16]. Quercetin, which is well absorbed in the small intestine, and rapidly metabolised to circulating sulfates and glucuronides in plasma[17,18], is the most abundant dietary flavonol, and recently actively studied in its vascular protection effects[5,19,20]. In the present study, we evaluated the role of quercetin metabolites (quercetin sulfate/glucuronide) on the prevention of apoptosis of HUVEC within the range of physiological concentrations[13,21].

Materials and methods

Preparation of quercetin sulfate/glucuronide

Quercetin sulfate/glucuronide was prepared from the serum of rats that were administered with quercetin and the

Abbreviations: DCFH-DA, 2′,7′-dichlorofluorescin diacetate; EGTA, ethylene glycol tetra-acetic acid; HUVEC, human umbilical vein endothelial cells; JNK, Jun N-terminal kinase; ROS, reactive oxygen species.

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concentration of quercetin sulfate/glucuronide was determined by HPLC after hydrolysis with sulfatase/glucuronidase as described previously[22,23]. The serum concentration of quercetin sulfate/glucuronide was 4·7 μM at the sampling time. Our quantification result indicated that the serum metabolites contained mainly sulfates and only a trace of glucuronides. There was no trace of quercetin aglycone.

Cell culture and cell treatment

HUVEC were cultured as previously described[2]. Cells were seeded at a density of 1×10^5 per 75 cm^2 flask in medium 199 (Gibco, Grand Island, NY, USA), supplemented with 20 mM-HEPES, endothelial cell growth substance (100 μg/ml) (Collaborative Research Inc., Waltham, MA, USA) and 20 % fetal calf serum (Gibco). The cultures were maintained at 37°C with a gas mixture of 5 % CO_2–95 % air. Subcultures were performed with trypsin–EDTA. All media were supplemented with heparin (5 U/ml), penicillin (100 IU/ml) and streptomycin (0·1 mg/ml). Medium was refreshed every third day. The endothelial cells were identified by the presence of factor VIII-related antigen (Histoset Kit; Immunolok, Carpinteria, CA, USA) and a typical 'cobblestone' appearance. Endothelial cells of the third to fifth passages in the actively growing condition were used for experiments. In experiments, HUVEC were treated with media containing 33 mM-glucose for different time intervals (24–48 h) in the presence or absence of ascorbic acid (100 μM) or quercetin sulfate/glucuronide (100 nM, 300 nM and 1 μM).

Detection of apoptosis

Apoptosis of treated HUVEC was detected by the ELISA method of cell death detection (Boehringer Mannheim, Indianapolis, IN, USA) as previously described[2]. For morphological assessment, cells were collected and fixed in a methanol–acetone (1:3, v/v) solution for 5 min and washed with PBS. Then fixed cells were stained with Hoechst 33 258 (0·1 ng/ml) for 10 min in the dark to counterstain nuclei. Cells were observed and photographed under a Nikon fluorescence microscope.

Detection of intracellular hydrogen peroxide production

Intracellular H_2O_2 production was monitored by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA) using 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Inc., Eugene, OR, USA)[2]. Briefly, cells (2×10^5/ml) were treated with medium containing 33 mM-glucose for the predetermined period and then co-incubated with 50 mM-DCFH-DA. After incubation, cells were re-suspended in ice-cold PBS and placed on ice in the dark for flow cytometry analysis.

Jun N-terminal kinase activity assay

Cell lysis and kinase activity assays were performed as previously described[2]. Briefly, after treatment with high glucose in the presence or absence of ascorbic acid or quercetin sulfate/glucuronide, cells were washed twice with ice-cold PBS and lysed in kinase buffer containing 20 mM-HEPES (pH 7·4), 50 mM-β-glycerophosphate, 1 % Triton X-100, 10 % glycerol, 2 mM-ethylene glycol tetra-acetic acid (EGTA), 1 mM-dithiothreitol, 10 mM-sodium fluoride, 1 mM-sodium orthovanadate, aprotinin (1 μg/ml), leupeptin (1 μg/ml) and 1 mM-phenylmethylsulfonyl fluoride. The soluble extracts were prepared by centrifugation at 14 500 rpm for 15 min at 4°C. After normalisation of protein concentration, the equal amounts of protein were incubated with protein A-sepharose and anti-JNK1 (1 μg; C17; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 3 h at 4°C. The immune complexes were washed twice with lysis buffer and then once with kinase assay buffer (20 mM-4-morpholinepropanesulfonic acid (pH 7·2), 2 mM-EGTA, 20 mM-$MgCl_2$, 1 mM-dithiothreitol and 0·1 % Triton X-100). The immune complexes were then re-suspended in 20 ml of kinase assay buffer containing 185 MBq of [α-^{32}P]ATP, 30 μM-cold ATP and 2 mg of glutathione S-transferase-c-Jun (1/79) as a substrate for JNK1, and incubated for 20 min at 30°C. Reaction was terminated by the addition of SDS sample buffer and boiling for 5 min. The protein was resolved by SDS-PAGE and visualised by autoradiography.

Caspase-3 activity assay

Caspase-3 activity was measured as described previously[2]. In brief, cells (1×10^6) were treated as indicated and the cytosolic extracts were prepared by repeated cycles of freezing and thawing in 300 μl of extraction buffer (12·5 mM-2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) (pH 7·0), 1 mM-dithiothreitol, 0·125 mM-EDTA, 5 % glycerol, 1 mM-phenylmethylsulfonyl fluoride, aprotinin (1 μg/ml) and leupeptin (1 μg/ml)). The cell lysates (100 μg) were diluted with the buffer (50 mM-Tris (pH 7·0), 1 mM-EDTA and 10 mM-EGTA) and incubated at 37°C with 10 mM-acetyl-Asp-Glu-Val-Asp-amino-4-methylcoumarin (Ac-DEVD-AMC), a caspase-3 substrate. The fluorescence of the cleaved substrate was measured by a spectrofluorometer (Hitachi F-3000; Hitachi, Tokyo, Japan) with an excitation wavelength at 380 nm and an emission wavelength at 460 nm.

Western blot analysis

Protein levels of JNK1 and caspase-3 were analysed by Western blot as described previously[2]. Briefly, the cell lysates were prepared, electrotransferred and then immunoblotted with anti-JNK1 and caspase-3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Detection was performed with Western blotting reagent ECL (Amersham, Centennial Avenue, Piscataway, NJ, USA) and chemiluminescence was exposed by the filters of Kodak X-Omat films.

Statistical analysis

Data were obtained from at least three separate experiments and presented as mean values and standard deviations. All statistical data were obtained by ANOVA followed by Student's *t* test. $P<0·05$ was considered statistically significant.

Results

Effect of quercetin sulfate/glucuronide on high glucose-induced apoptosis

High glucose induced apoptosis of HUVEC in a time-dependent manner that could be effectively inhibited by ascorbic acid

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(100 μM) and quercetin sulfate/glucuronide (300 nM) (Fig. 1(a)). Apoptosis induced by high glucose at 48 h was inhibited by quercetin sulfate/glucuronide (100 nM, 300 nM and 1 μM) in a dose-dependent manner. The inhibitory effect of quercetin sulfate/glucuronide (300 nM) on high glucose-induced apoptois was similar to that of ascorbic acid (100 μM) (Fig. 1(b)).

Effect of quercetin sulfate/glucuronide on high glucose-induced reactive oxygen species generation

Flow cytometry was used to investigate the amount of H_2O_2 that was generated in high glucose-induced apoptosis of HUVEC. Treatment with high glucose was found to increase DCFH fluorescence at 48 h, which was completely suppressed by quercetin sulfate/glucuronide at the concentration of 300 nM (Fig. 2).

Effect of quercetin sulfate/glucuronide on high glucose-elicited Jun N-terminal kinase activity

In our previous study[2], JNK activity in high glucose-treated HUVEC was identified to increase at 24 h and sustain up to 48 h. In the present study, after 48 h treatment of high glucose in the presence of quercetin sulfate/glucuronide (100 nM, 300 nM and 1 μM), the increase of JNK activity could be reversed in a dose-dependent manner (Fig. 3 (a)). The inhibitory effect of quercetin sulfate/glucuronide (300 nM) on JNK activity was similar to that of ascorbic acid (100 μM) (Fig. 3 (b)). Quercetin sulfate/glucuronide alone did not alter JNK activity.

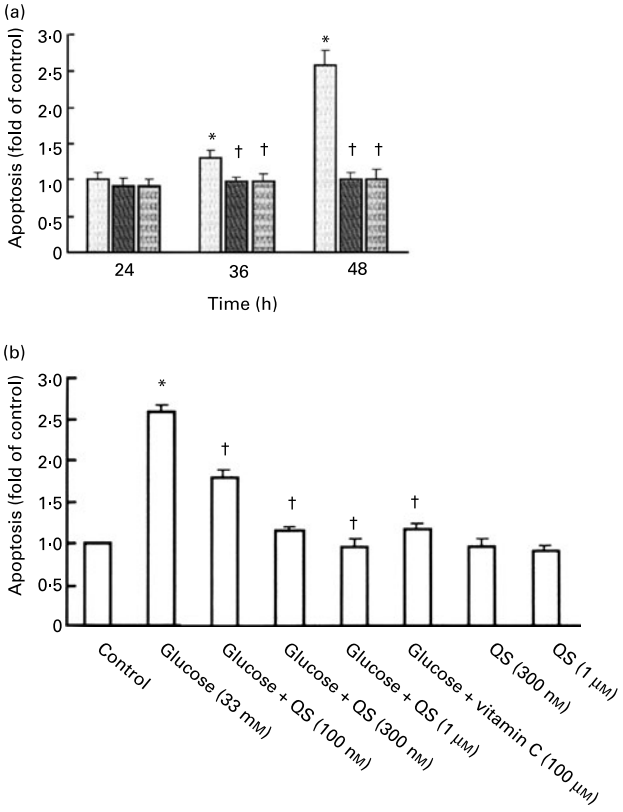

Fig. 1. (a) Time-dependent high glucose-induced apoptosis in human umbilical vein endothelial cells (HUVEC). HUVEC were treated with high glucose (33 mM; □) for 24 to 48 h in the presence or absence of ascorbic acid (vitamin C; 100 μM; ■) or quercetin sulfate/glucuronide (QS; 300 nM; ▨). Cell death was detected by ELISA as described in the Methods. (b) Dose-dependent effect of QS on high glucose-induced apoptosis in HUVEC. HUVEC were treated with high glucose (33 mM) for 48 h in the presence or absence of vitamin C (100 μM) or QS (100 nM, 300 nM and 1 μM). The inhibitory effect of QS (300 nM) on apoptosis was similar to that of vitamin C (100 μM). Data are means, with standard deviations represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0·05$). † Mean value was significantly different from that of the group with high glucose treatment ($P<0·05$).

Fig. 2. High glucose-induced intracellular H_2O_2 formation in human umbilical vein endothelial cells (HUVEC). HUVEC were treated with high glucose (33 mM) for 48 h in the presence or absence of quercetin sulfate/glucuronide (QS; 300 nM). Intracellular H_2O_2 production was determined by the fluorescence of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as described in the Methods. Data are means, with standard deviations represented by vertical bars. * Mean value was significantly different from that of the control group ($P<0·05$). † Mean value was significantly different from that of the group with high glucose treatment ($P<0·05$).

Effect of quercetin sulfate/glucuronide on high glucose-elicited caspase-3 activity

In our previous study[2], caspase-3 activity, determined in high glucose-treated HUVEC, was shown to increase with time up to 48 h and could be inhibited by ascorbic acid (100 μM). In the present study, after 48 h treatment of high glucose, quercetin sulfate/glucuronide (300 nM and 1 μM) was demonstrated to suppress caspase-3 activity effectively (Fig. 4). Quercetin sulfate/glucuronide alone did not alter caspase-3 activity.

Discussion

Endothelial dysfunction is an early manifestation of vascular atherosclerosis and also an independent predictor of poor prognosis in CVD[6]. Diabetes mellitus is an important risk factor for CVD[1]. Research has revealed that high glucose would induce production of reactive oxygen species (ROS), which can cause endothelial dysfunction and even cell apoptosis[2–5]. Our previous studies demonstrated that the high glucose-induced ROS could activate c-JUN N-terminal protein kinase (JNK), which leads to triggering of caspase-3 and facilitation of apoptosis in human endothelial cells[2,3]. Experimental studies have shown that vitamin C is a strong antioxidant and prevents ROS-mediated vascular dysfunction and apoptosis at high or above physiological concentrations[7,8,12,13]. However, most of the clinical observational

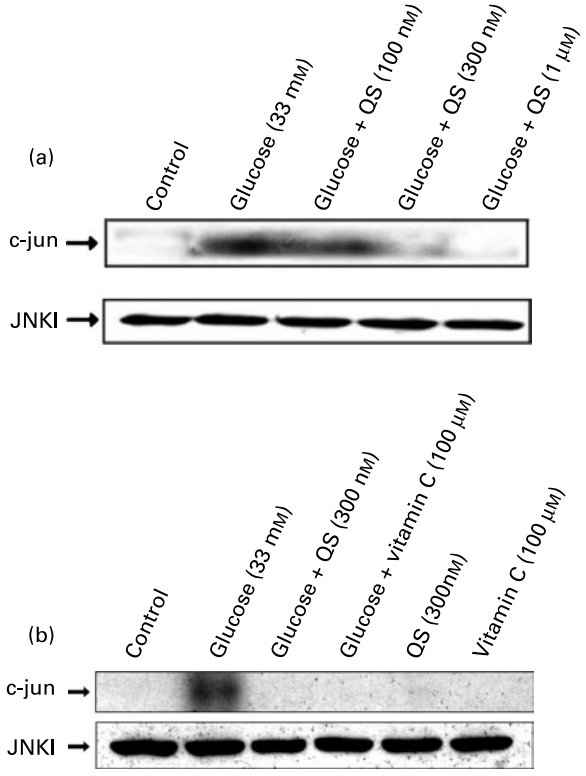

Fig. 3. (a) Dose-dependent effect of quercetin sulfate/glucuronide (QS) on high glucose-induced Jun N-terminal kinase (JNK) activity. Human umbilical vein endothelial cells were treated with high glucose (33 mM) for 48 h in the presence or absence of QS (100 nM, 300 nM and 1 μM). The kinase activity assay was performed by immunocomplex assay (top) and the protein level was determined by Western blot (bottom), as described in the Methods. (b) Comparison of the effect of QS and vitamin C on JNK activity. The inhibitory effect of QS (300 nM) on JNK activity was similar to that of vitamin C (100 μM).

and prospective studies failed to show cardiovascular benefits from vitamin C therapy[9–11]. Recently, dietary flavonoids have received much attention, since epidemiological studies report an inverse association between dietary flavonoid consumption and mortality from CVD[15,16]. Many experimental data have accumulated regarding the protective effects of flavonoids on the endothelium[14]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone), one of the most abundant flavonoids in the human diet, has been known as an antioxidant and represents the most prototypical example of flavonoids[5,19,20]. Quercetin is well absorbed in the small intestine and further metabolised to circulating sulfates and glucuronides[17,18]. Therefore, in view of the potential of quercetin metabolites as a pharmacological agent, it would be of interest to investigate the effect of quercetin sulfate/glucuronide within the range of physiological concentrations on ROS and the possible involvement of the signalling pathway in high glucose-induced apoptosis of human endothelial cells[13,21].

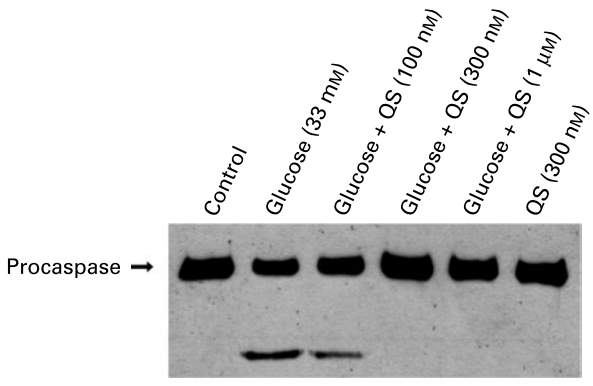

Fig. 4. Dose-dependent effect of quercetin sulfate/glucuronide (QS) on high glucose-induced caspase-3 activity. Human umbilical vein endothelial cells were treated with high glucose (33 mM) for 48 h in the presence or absence of QS (100 nM, 300 nM and 1 μM). Western blotting to identify caspase-3 was performed as described in the Methods. Caspase-3 cleavage products were seen 48 h after treatment with high glucose. QS (300 nM and 1 μM) suppressed caspase-3 activity effectively.

Previous studies revealed that polyphenolic flavonoids, including quercetin, exerted protective effects *in vitro* against apoptosis mediated by H_2O_2 and oxidised LDL in HUVEC at the doses between 10 μM and 50 μM[5,20,24]. In the present study, we investigated the effect of quercetin sulfate/glucuronide on high glucose-induced apoptosis and found that the anti-apoptotic effect of quercetin sulfate/glucuronide was dose-dependent and remained significant at the nanomolar concentrations (300 nM and 100 nM). The effective concentrations of quercetin sulfate/glucuronide used in the present experimental study were within the range of quercetin concentrations detected in humans[13,21]. The single concentration of ascorbic acid (100 μM) chosen for reference in the present study was based on the experience of our previous study[2] and the study conducted by Rössig *et al.* [7], which showed that the effective concentration of ascorbic acid on inhibiting TNF-α (50 ng/ml)-induced apoptosis in HUVEC was 100 μM, but not 10 μM and 50 μM. Nevertheless, in the present study, we did not evaluate the dose-dependent effect of ascorbic acid on apoptosis and would not attempt to jump to a conclusion that quercetin metabolites rather than vitamin C could have superior cardiovascular benefits in clinical studies.

High glucose may activate NADPH oxidase and lead to the production of ROS, including the superoxide anion and H_2O_2[25,26]. Recently, quercetin was found to down-regulate NADPH oxidase and prevent endothelial dysfunction in spontaneously hypertensive rats[27]. Although previous studies have shown that quercetin in the low micromolar range is an effective scavenger of H_2O_2[20,24], the antioxidant effect of quercetin sulfate/glucuronide in the nanomolar range on high glucose-treated HUVEC has not yet been clarified. In the present study, we demonstrated that the low concentration of quercetin sulfate/glucuronide (300 nM) could also completely suppress the increase of DCFH fluorescence, which is used for detection of intracellular H_2O_2 induced by high glucose.

In the present study, quercetin sulfate/glucuronide (300 nM) was also found to inhibit JNK activity effectively. In different cell types, however, there were discrepant effects of quercetin on cell viability via inhibition of JNK activity. In HUVEC, quercetin showed little cytotoxicity at 100 μM, and inhibited JNK activity with consequent prevention of apoptosis in the low micromolar range (10 μM to 50 μM)[20]. In contrast, quercetin at 100 μM induced apoptosis of intimal-type rat vascular smooth muscle cells via the inhibition of JNK activity[28]. It is not clear why quercetin has either an anti-apoptotic or pro-apoptotic effect on different cells. However, either anti-apoptosis of HUVEC or pro-apoptosis of intimal vascular

smooth muscle cells plays an important role in the anti-atherosclerotic effect of quercetin.

Quercetin has been demonstrated to prevent H_2O_2-mediated and LDL-induced apoptosis of HUVEC via the inhibition of caspase-3 in the low micromolar range (10–50 μM)[5,20,24]. In the present study, we showed that even lower doses of quercetin sulfate/glucuronide (300 nM and 1 μM) effectively suppressed caspase-3 activity. The dose-dependent inhibitory effect of quercetin sulfate/glucuronide on H_2O_2 production, JNK and caspase-3 activity was consistent.

Previous studies have shown that quercetin-3′-sulfate and quercetin-3-glucuronide are the two major quercetin conjugates in human plasma, but the ratio of them may vary between individuals[18,29]. Other studies have also reported that quercetin-3′-sulfate and quercetin-3-glucuronide can exert different or opposing effects on variant endothelial cells[30–32]. The opposing properties of the coexisting natural compounds have been postulated as the existence of the yin-yang paradigm[33], and the balance of circulating quercetin conjugates has been suggested to determine the final effect *in vivo* [31]. In the present study, we were not able to indicate the substitute positions of the metabolites in the study because they were a complex mixture of sulfates and glucuronides. Our quantification result revealed that the serum metabolites contained mainly sulfates and only a trace of glucuronides. However, we also believe that the effect exerted by this metabolite combination prepared from rats mimicked the *in vivo* situation more than a pure compound.

In conclusion, we showed for the first time that the effect of quercetin sulfate/glucuronide on H_2O_2 quenching, inhibition of JNK and caspase-3 activity was effective at a nanomolar concentration. The findings of the present study may be useful and shed light on the pharmacological application of quercetin metabolites (quercetin sulfate/glucuronide) in CVD.

Acknowledgements

The present study was supported by grants (PTH9707 and PTH9709) from Taoyuan General Hospital, Department of Health, the Executive Yuan, Taiwan. We are grateful to Ms Chia-Chi Chen and Mr Shr-Ping Chou for their technical assistance in the present study.

The present study was supported by grants (PTH9707 and PTH9709) from Taoyuan General Hospital, Department of Health, the Executive Yuan, Taiwan. The authors C.-L. C., C.-S. W. and F.-M. H. conducted the design and implementation of the experiments, and the completion of the manuscript. Y.-C. H. and P.-D. L. C. contributed to the preparation of quercetin sulfate/glucuronide. All authors read and approved the submission of this paper. There are not any conflicts of interest in the submission of this paper.

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British Journal of Nutrition