The effects of chronic *trans*-resveratrol supplementation on aspects of cognitive function, mood, sleep, health and cerebral blood flow in healthy, young humans.

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ABSTRACT

Single doses of resveratrol have previously been shown to increase cerebral blood flow (CBF) with no clear effect on cognitive function or mood in healthy adults. Chronic resveratrol consumption may increase the poor bioavailability of resveratrol or otherwise potentiate its psychological effects. In this randomised, double-blind, placebo-controlled, parallel-groups study a total of 60 adults aged between 18-30yrs received either placebo or resveratrol for 28 days. On the 1st and 28th day of treatment the performance of cognitively demanding tasks (Serial subtractions, Rapid Visual Information Processing and 3-Back) (N= 41 complete datasets) were assessed, alongside blood-pressure (N= 26) and acute (Near-infrared Spectroscopy [NIRS]) and chronic (Trans-Cranial Doppler [TCD]) measures of CBF (N= 46). Subjective mood, sleep quality and health questionnaires were completed at weekly intervals (N= 53/54). The results showed that the cognitive effects of resveratrol on day 1 were restricted to more accurate but slower Serial Subtraction task performance. The only cognitive finding on day 28 was a beneficial effect of resveratrol on the accuracy of the 3-Back task prior to treatment consumption. Subjective ratings of 'fatigue' were significantly lower across the entire 28 days in the resveratrol condition. Resveratrol also resulted in modulation of CBF parameters on day 1, as assessed by NIRS, and significantly increased diastolic BP on day 28. Levels of resveratrol metabolites were significantly higher both before and after the day's treatment on day 28, in comparison to day 1. These results confirm the acute CBF effects of resveratrol and the lack of interpretable cognitive effects.

INTRODUCTION

Resveratrol (*3, 4', 5 trihydroxystilbene*) is a polyphenolic secondary metabolite produced within plants in response to a range of environmental stressors[1]. Previous investigations in young, healthy humans have demonstrated significantly increased cerebral blood flow (CBF) after acute resveratrol supplementation[2] which is likely mediated by the ability of resveratrol to modulate nitric oxide (NO) synthesis[3]. In line with this, oral consumption has been shown to enhance endothelium-dependent relaxation in rats[4, 5], and improve flow-mediated dilatation in overweight/obese humans[6]. An increase in blood-borne neural metabolic substrates such as oxygen[7] and glucose[8] have been shown to enhance aspects of cognitive performance in healthy, young humans. However, to date there is no evidence that cognitive function is modulated during acute, resveratrol-mediated increases in CBF.

One potential explanation for this lack of cognitive effects is the rapid metabolism and poor bioavailability of oral resveratrol[9] which might reduce its potential bioactivity. Pharmacokinetic studies have demonstrated plasma C_{max} levels of resveratrol metabolites between 0.9-3.7 μM following a single oral dose of 500mg resveratrol[10] with levels of the parent compound at trace, or undetectable concentrations[2, 10-13] after acute, bolus supplementation. Conversely, results from 3 preclinical chemopreventive efficacy papers suggest that repeated low daily doses of resveratrol (up to 2mg/kg) are sufficient to produce peak plasma concentrations of aglycone resveratrol of up to 2μM, potentially exerting beneficial chemopreventive effects[14] possibly as a result of a cumulative increase in plasma levels of resveratrol.

Thus the current study investigated the effects of 28 day supplementation with 500mg resveratrol in healthy adults with the hypothesis being that daily consumption of this polyphenol, over an extended period, may increase bioavailability in terms of plasma levels, and potentiate any effects on cognitive performance and CBF. In the current study continuous wave (CW) Near-Infrared Spectroscopy (NIRS) was utilized to monitor acute changes in CBF in the prefrontal cortex during the performance of cognitive tasks that activate this brain region. This technique was combined with Trans-cranial Doppler sonography (TCD), applied to the middle cerebral artery (MCA), which provides a measure of acute and chronic changes in global CBF velocity (CBFV) and which has been converged successfully with NIRS previously [15]. Resveratrol has previously been shown to interact with a number of diffuse, health related parameters such as antioxidant and anti-inflammatory status[16, 17], monoamine oxidase-A and B activity[18] and Peroxisome proliferator-

activated receptor gamma coactivator 1-alpha PGC-1α[19] production. Hence, the current study also assessed health, mood and sleep parameters via questionnaires.

EXPERIMENTAL METHODS

Participants

All participants reported themselves to be in good health and free from illicit drugs, alcohol, prescription medication and herbal extracts/food supplements at each assessment. Participants confirmed that they would also abstain from the latter for the duration of the study and that any changes in medication or health status would be reported to the researcher when they occurred. Participants who had suffered a head injury, neurological disorder or neuro-developmental disorder were excluded from participation, as were those who did not have English as their 1st language, or had any relevant food allergies or intolerances, digestive problems, smoked tobacco, drank excessive amounts of caffeine (more than 600mg/day as assessed by a caffeine consumption questionnaire), took illicit social drugs, were pregnant, seeking to become so, or were breast feeding.

The study received ethical approval from the Northumbria University Psychology department (within the Faculty of Health and Life Sciences) ethics committee (reference: SUB16_EW_1010; date approved 11/11/2010) and was conducted according to the Declaration of Helsinki (1964). All participants gave their written informed consent prior to their inclusion in the study.

See table 1. for participant composition (broken down per analysis).

Table 1. Participant composition. Table displays number of participants included in each measure. Sixty participants were originally recruited to take part in all aspects of assessments apart from the blood pressure measurement which utilized only 30 participants due to the potential disruption this may have caused to NIRS measurement. Reasons for excluding data from analyses include: technical problems with equipment (affecting aspects of 12 cognitive performance data sets, 14 NIRS, 14 TCD recordings (namely not being able to locate a consistent, 5 minute, blood flow trace in the latter and data which was outside of the calculated standard deviations of this cohort, and may suggest an ill-fitting headband, with regards NIRS) and 6 blood pressure readings) and participants not complying with proper completion of measures/ omitting to respond (affecting aspects of 7 cognitive performance data sets, 7 responses from the GHQ, 6 from the POMS, 7 from the PSQI, 5 from the food consumption questionnaire and 3 from the treatment guess response).

Measure (Number participants)	Female/Male	Mean age (Age range)	Right handed/ Left handed	Placebo/ Resveratrol
Overall recruited (N=60)	51/9	20.52 (18-29)	53/7	30/30
Cognitive performance (N=41)	36/5	20.00 (18-27)	35/6	19/22
NIRS (N=46)	39/7	20.45 (18-29)	39/7	24/22
TCD (N=46)	40/6	20.08 (18-29)	40/6	21/25
Blood pressure (N=24)	21/3	20.75 (18-29)	21/3	15/9
GHQ (N=53)	45/8	20.17 (18-29)	46/7	28/25
POMS (N=54)	46/8	20.07 (18-29)	47/7	28/26
PSQI (N=53)	45/8	20.15 (18-29)	47/6	28/25
Food consumption (N=55)	47/8	20.15 (18-29)	48/7	29/26
Treatment guess (N=57)	49/8	20.25 (18-29)	50/7	28/29

Treatments

Over the course of this 28 day supplementation study, participants received either 500mg pure *trans*-resveratrol (Transmax™ by Biotivia™ with a guaranteed purity of 98%. Also containing 10mg piperine per capsule), or an inert placebo (methyl cellulose), once daily; with the treatment allocation dictated by Latin square. Participants were instructed to consume their daily capsule in the morning and preferably with breakfast.

Participants consumed their first and last capsule of treatment during the two lab visits and were instructed to self-supplement every day in the interim. Participants kept a treatment log during this time, noting down the time of capsule consumption every day. A treatment pot containing 32 capsules was given to each participant at the end of visit 1- enough for 28 days of supplementation plus extra in case of loss/continued supplementation due to unforeseen circumstances/and to verify compliance.

All treatments were administered in identical green vegetarian capsules with the Biotivia™ logo and presented in identical white treatment pots with only the participant number to identify them. All treatments were produced by Biotivia™, prepared by the lead investigator and coded by a third party who had no further involvement in any aspect of the study. No member of the investigational team was aware of the contents of the capsules until a blind-data review was completed.

Measures of cerebral-blood flow (CBF)

Two complementary techniques were utilised:

Acute changes in CBF - Near-Infrared spectroscopy (NIRS)

NIRS is non-invasive brain imaging technique predicated on the absorption by oxygenated and deoxygenated haemoglobin of differing wavelengths of infra-red light, introduced through the intact scalp/skull. Continuous-wave NIRS (CW-NIRS) can be used to assess acute changes in local CBF, as indexed by concentration changes in total haemoglobin during a single continuous recording session, See[2] for full description of the methods employed here. Given that CW-NIRS generates concentration change data that is intrinsically baseline-adjusted to the concentration immediately prior to the first data point in the recording session, it cannot be used to quantify gross changes in CBF parameters that take place between two separate recording sessions. In this instance the change from baseline data generated by the NIRS system was subjected to a second baseline adjustment by creating 'change from baseline' data with respect to the 10 minutes of NIRS data collected immediately prior to the treatment- this provided a more accurate baseline measure of immediately pre-treatment NIRS parameters. All subsequent NIRS data was collapsed into 2 minute epochs (20 resting period epochs spanning 0- 40 min and 20 task period epochs spanning 40- 80 min).

Chronic changes in CBF - Trans-cranial Doppler sonography (TCD)

Given the inability of CW-NIRS to measure chronic changes in CBF parameters, a second measure of CBF was also employed. Trans-cranial Doppler sonography (TCD) is a non-invasive method of measuring cerebral blood flow velocity (CBFV) through the basal intracerebral vessels through the intact skull[20] and was utilized at pre- and post-dose time points on day 1 and day 28. Pulses of ultrasound penetrate the skull at a number of 'acoustic windows', which include: temporal, orbital, foraminal and submandibular, insonating vessels at particular depths, with the returning 'echo' displayed as a Doppler waveform[21]. The mean velocity, peak systolic velocity, diastolic velocity, and pulsatility index (all cm/sec) of the insonated vessel are provided; indicating the speed of the flow of blood and the variability of blood velocity.

TCD has been utilized to investigate blood flow abnormalities in a number of haematological; e.g. stroke risk in sickle cell patients[22], and vascular; e.g. cerebrovascular reactivity in degenerative and vascular dementia[23], disorders as well as investigating the relationship between brain activity (in response to cognitive tasks) and blood flow velocity in healthy participants[24] and the CBFV response to pharmacological interventions; e.g. caffeine[25] and drugs; e.g. in cocaine abusers[26].

In the current study, CBFV was measured with participants sitting in a reclined position in a quiet room. A trans-temporal acoustic window was utilized for assessment of the right middle cerebral artery (MCA) using pulsed TCD (Digi-Lite™, RIMED) with a 2MHz probe held in place by a light, mounted head frame. This device provides mean velocity, peak systolic velocity, diastolic velocity, and pulsatility index information every 30 seconds; equating to ~10 values across the 5 minute recording utilized here, for each of the 4 aforementioned variables. These were averaged to give 1 value for that time-point (pre- and post-dose on day 1 and pre- and post-dose on day 28) for statistical analysis.

Cognitive tasks

The computerised battery of cognitive tasks (which all, to a greater or lesser extent, activate the prefrontal cortex: Serial subtractions[27]; RVIP[28]; 3-Back[29]) were delivered on a laptop using the Computerised Mental Performance Assessment System (COMPASS, University of Northumbria) software, and comprised:

Serial subtractions (2 mins each of serial 7s, 13s and 17s):

Rapid Visual Information Processing [RVIP] (2 mins):

Both the serial subtraction and RVIP task are described in detail in[2].

3-back: The 3-back version of this task was used in this paradigm, requiring participants to indicate whether the letter presented on screen was also present 3 letters back in the letter sequence. Participants must respond by pressing the 'yes' or 'no' button on the response box, to each letter, as quickly as they can. This task lasts for 2 minutes and is scored for accuracy and reaction time.

Questionnaires

Food consumption questionnaire:

A non-validated food consumption questionnaire was utilized to collect information on the general diet of participants (e.g. 'How many portions of fruit and vegetables did you eat on an average day in the past week?') and specifically polyphenol/resveratrol consumption (e.g. 'In the entire previous week, on how many occasions have you eaten a portion of berries or grapes?'). The questionnaire consisted of 13 questions with several also relating to compliance (e.g. 'Was treatment consumed with breakfast and/or before 9:30am every day in the past week?') and medication ('Have you

consumed any medication in the past week? If so, please state the medication, dose, when taken and for what reason.'). This researcher-created questionnaire has no reliability/sensitivity measures and was utilized solely as a tool to detect any gross changes in the consumption patterns of participants which might affect outcome measures. The researcher noted no salient dietary or medication changes across the study for any of the participants.

General Health Questionnaire (GHQ):

The GHQ[30] utilized in the current study was the 28-item scaled version which assesses somatic symptoms, anxiety and insomnia, social dysfunction and severe depression. The 28 items are scored from 0-3 with participants indicating the frequency or extent to which they have experienced a number of issues, such as 'Have you recently been having hot or cold spells?', in the previous week. The items combine to assess the 4 aforementioned sub-scales and the total possible score (when these 4 sub-scales are collated) ranges from 0- 84; with higher scores representing more negative symptoms.

Profile Of Mood States (POMS):

The POMS is a well validated questionnaire of mood states and their fluctuations both in the clinical and research setting[31]. Participants rated 65 adjectives (e.g. unhappy, considerate), in terms of how much they had felt each one in the past week, utilizing a 5-point scale from 'not at all' to 'extremely'. Scores from these 65 items (which includes 7 dummy adjectives) are combined to give 6 global scores of 'tension', 'depression', 'anger', 'fatigue', 'confusion' and 'vigour'. A total mood disturbance score can also be calculated by adding the scores from the first 5 of these global scores and subtracting 'vigour'.

Pittsburgh Sleep Quality Inventory (PSQI):

The PSQI is a well validated subjective measure of the quality and pattern of sleep[32]. The current study tailored this questionnaire to assess sleep during the past 'week' rather than 'monthly' as per the original. The PSQI assesses 7 factors: subjective sleep quality; sleep latency; sleep duration; habitual sleep efficiency; sleep disturbances; use of sleep medication and daytime dysfunction, via questions regarding sleep timings and 0-3- point scales where participants rate whether they have experienced a number of issues (e.g. 'During the past week, how often have you had trouble sleeping because you have had bad dreams?') from 'not during the past week' to '3 or more times in the past week'. A global sleep score is created by totalling the 7 sub-factor scores with higher scores indicating poorer sleep quality.

Treatment guess

During the day 28 visit participants were asked to guess which treatment they thought they had been taking for the duration of the study and to explain any reasons for that guess.

Procedure

This investigation required participants to attend the laboratory for an initial training/ screening session and then on 2 separate occasions, 28 days apart, for laboratory-based testing sessions. Participants were required to supplement themselves with 1 capsule per day in the interim.

Upon arrival at both day 1 and day 28 lab visits participants completed 4 questionnaires: a food consumption questionnaire; the GHQ; POMS; and the PQSI. All questionnaires were answered in relation to the previous 7 days and completed every 7 days during the supplementation period. After filling in the questionnaires, participants then gave a blood pressure reading or an intravenous blood sample (15 participants provided blood samples- see below for more information and the demographics of the 7 participants from the resveratrol condition entered into the analysis) which was immediately followed by a 5 minute rest. A 5 minute recording of cerebral perfusion in the MCA was then taken with TCD. The NIRS headband was then positioned onto the forehead of the participant to monitor CBF in the prefrontal cortex throughout the session. Once a reliable trace was identified participants commenced 20 minutes (x2 repetitions of the battery) of baseline cognitive tasks. The first of these repetitions acted as a 'refresher', attenuating any practice effects, and the second was utilized to create change from baseline data for the analysis of cognitive outcome data. A 10 minute rest period then followed with NIRS data averaged across this period and used as an accurate, immediately pre-treatment baseline for the calculation of change from baseline data for the post-treatment periods. During this 10-min resting period participants watched a non-arousing DVD. Participants then consumed the first day's treatment and continued to watch the DVD for a further 40 minute absorption period. After this period a blood pressure reading was taken in those who did not provide a blood sample previously and 40 minutes of post-dose tasks commenced. After task completion a further blood pressure reading was taken from the relevant participants and followed by a short break before the 2nd TCD recording was conducted. Following the TCD recording participants were either free to leave the lab or provided a final blood sample if they were part of the aforementioned sub-section of participants. The timelines and running order of the testing sessions are shown in figure 1.

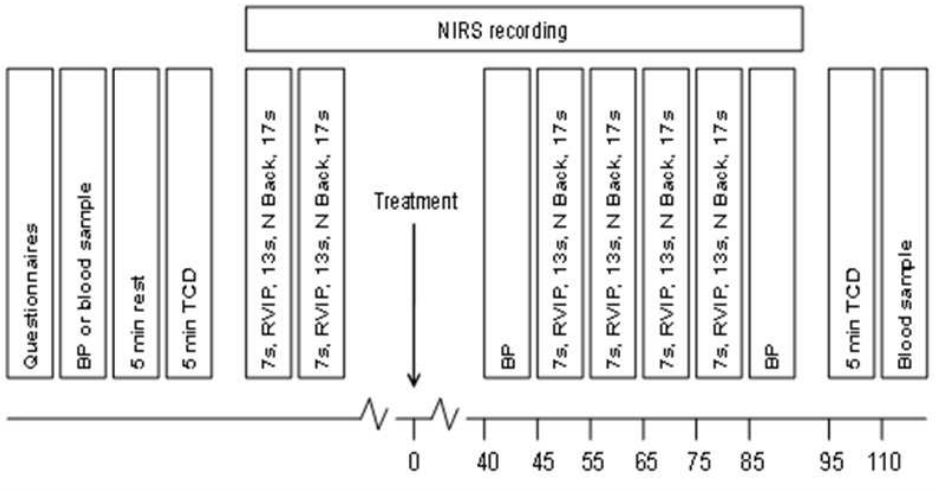

Figure 1. Day 1 and 28 esting session timeline. Upon arrival participants completed 4 questionnaires (a food consumption questionnaire, the GHQ, POMS and the PQSI) which they answered in relation to the previous 7 days and completed every 7 days during the supplementation period. Participants then gave a blood pressure reading or an intravenous blood sample which was immediately followed by a 5min rest. A 5min recording of cerebral perfusion in the MCA was then taken with the TCD. The NIRS headband was then positioned and 20min of baseline tasks commenced. A 10min rest then followed during which participants watched a non-arousing DVD. Participants then consumed their treatment capsule and continued to watch the DVD for a further 40min absorption period. A blood pressure reading was then taken from a sub-sample of participants and 36mins of post-dose tasks commenced. The NIRS headband was removed and a further blood pressure reading taken, followed by a short break, before the 2nd TCD recording was conducted. Following the TCD recording the aforementioned sub-section of participants provided a blood sample and left the lab.

Bioavailability assessment

Participants:

Complete sample sets comprising all 4 time-points were obtained from 15 participants (8 from placebo and 7 from resveratrol; 10 females, 5 males; mean age 19.87 years; range 18-25 years). All participants were asked, at the beginning of the study, if they would provide blood samples as part of the investigation: the above 15 participants represent those who agreed to this aspect of the study and for whom all 4 samples could be collected in full. The 7 resveratrol participants included in the analysis comprised: 6 females, 1 male; mean age 19.43 years, range 18-21 years.

Venous blood samples were collected before the days treatment was consumed and 110-minutes post-dose in this sub-sample of participants using 4.7ml monovettes (Sarstedt AG & Co) containing lithium heparin. Samples were centrifuged at 2500rpm for 15 minutes at 20^{o}C to yield plasma, which was then stored at -80^{o}C until analysis.

The preparation of Samples and LC-MS analysis is as per a previous study conducted by this lab [33].

Statistics

The analyses of TCD, plasma, questionnaire, behavioural and treatment guess data were conducted with IBM SPSS Statistics 19.0 for Windows (SPSS Inc, Chicago, IL). NIRS data was analysed with Minitab 16 for Windows (Minitab Inc, State College, PA).

Questionnaire data analysis:

Questionnaire data (GHQ, POMS and PSQI) for each of the four post-dose weekly completions was analysed as change from baseline (the questionnaire scores obtained on day-1 prior to treatment) for each individual variable/sub-component by a mixed (Day (x4): 7, 14, 21, 28, by Treatment (x2): 500mg resveratrol and placebo) ANOVA with Bonferroni corrected post-hoc student t tests conducted if a significant main and/or interaction effect was evinced here.

Treatment guess analysis:

Treatment guess data was analysed by Chi-Square.

Trans-cranial Doppler (TCD):

The raw data for each of the four TCD variables (Mean Velocity, Peak Systolic Velocity, Diastolic Velocity and Pulsatility Index) were analysed by a mixed (Treatment (x2): 500mg resveratrol and placebo, by time (x4): baseline day 1, post-dose day 1, pre-dose day 28 and post-dose day 28) ANOVA.

Plasma analysis:

The raw data for each of the four forms of plasma resveratrol (resveratrol-3-sulfate, resveratrol-4-glucuronide, resveratrol-3-glucuronide and 'total metabolites'; which is the sum of the three metabolites) was analysed via ANOVA with time as a factor (x4: baseline day 1, post-dose day 1, pre-dose day 28 and post-dose day 28).

Cognitive task data and Blood Pressure (BP) analysis:

The cognitive task and BP measures produce data that can be analysed to assess both acute (potential treatment effects within day 1), pure-chronic (chronic treatment-related effects which have taken place across the 28 day supplementation period but prior to taking the day 28 treatment) and superimposed acute/chronic (the difference in 'acute' effects between day 1 and day 28) effects

of resveratrol. In order to adequately analyse the 'acute', 'pure chronic' and 'superimposed acute/chronic' effects of the treatments 2 separate ANOVAs were conducted:

1. Pure chronic effects:

To ascertain if any pure chronic effects of resveratrol supplementation had taken place, pre-dose data on day 28 was converted to change from day 1 pre-dose baseline and analysed via one-way ANOVA to compare performance between treatments.

2. Acute, chronic and superimposed effects:

To ascertain if any acute and/or superimposed chronic effects of resveratrol supplementation had taken place, data was converted to change from baseline with respect to the pre-treatment scores on the first day of treatment (day 1) and analysed via a repeated measures ANOVA (treatment (resveratrol/ placebo, X repetition (x4 for cognitive data and x2 for BP), by day (day 1/28).

Both ANOVAs were utilized in order to tease apart acute effects restricted to day 1 (treatment x day interactions with significant effects restricted to day 1), acute effects across both day 1 and day 28 (main effect of treatment and/or a treatment x repetition interaction) and a superimposed acute/chronic effect (treatment x day interaction with significant effects restricted to day 28 (interpreted with reference to the pure chronic ANOVA results)). If any such main and/or interaction effects were observed then Bonferroni corrected post-hoc student t tests were conducted to assess where these differences lie. This analysis plan has proven sensitivity in detecting the acute and chronic effects of ginseng in healthy, human participants previously[34].

Near-Infrared Spectroscopy analysis:

NIRS data was converted to 'change from baseline' (calculated from the 10 minute pre-treatment resting period) and averaged across 2 minute epochs during the 40 minute 'rest/absorption' and 40 minute cognitive task performance period. Analysis of variance (treatment group x 2min epoch x day) was conducted on this data with planned comparisons of data from each epoch being made between placebo and 500mg resveratrol (resulting in 40 planned comparisons for oxy-HB, Deoxy-Hb and total-Hb) using t tests calculated with the Mean Squares Error from the ANOVA[35]. A significant result on this ANOVA was not used as a prerequisite for carrying out and interpreting the planned comparisons and are, therefore, not presented here. However, in order to reduce the potential for Type I errors, all planned comparisons were Bonferroni corrected and only those planned comparisons associated with a consistent pattern of significant effects are interpreted and reported herein.

RESULTS

Compliance

Potential compliance ranged from 0-114% (the upper limit reflecting 32 capsules consumed over 28 days). Average compliance was 101% with a range of 78.5-114.3%. Data from one participant with 78.5% compliance (who provided blood samples in the placebo condition only) was excluded from analysis, due to being below a pre-set level of 80%, making average compliance 101.4% with a range of 92.9%-114.3%.

Treatment guess

Chi-Square revealed no significant difference between treatment guesses in the 2 treatment groups: χ^2= .766; df= 1; p= .381.

NIRS parameters

Total haemoglobin (total-Hb):

Planned comparisons revealed that, on day 1, levels of total-Hb were significantly higher after resveratrol, compared to placebo, during the 2-minute epochs spanning 35-38 min post-dose (35/36min [p=0.003], 37/38 min [p=0.008]) of the absorption period and the epochs spanning 75-78 min (75/76 min [p=0.008], 77/78 min [p=0.005] of the post-dose task period. No significant differences were found between resveratrol and placebo on day 28.

Oxygenated haemoglobin (oxy-Hb):

Planned comparisons revealed that, on day 1, levels of Oxy-Hb were significantly higher in the resveratrol condition, compared to placebo, during the 2-min epochs commencing 23 [p=0.002], 27 [p=0.005], 33 [p=0.002], 35 [p=0.001] and 37 [p=0.009] min post-dose of the absorption period and the epochs spanning 41-44 mins (41/42 min [p=0.006]), 43/44 min [p=0.001]), 53-54 min [p=0.001], 61-68 min [p= 0.0008; 0.001; 0.007 and 0.001 respectively], 71-72 min [p=0.003], and 75-78 min (75/76 min [p=0.0002], 77/78 min [p=0.0002]) of the post-dose task period. No significant differences were found between resveratrol and placebo on day 28.

Deoxygenated haemoglobin (deoxy-Hb):

Planned comparisons revealed that, on day 1, levels of deoxy-Hb were significantly higher in the placebo condition, compared to resveratrol, during the 2-minute epochs commencing 27 [p=0.001], 29 [p=0.006] and 35 [p=0.003] min post-treatment in the absorption period and the epochs commencing 43 min [p=0.004] min, and spanning 51-54 min (51/52 min [p=0.0002], 53/54 min [p=0.004], and those spanning 61-72 min (61/62 [p=0.001], 63/64 [p = 0.003], 65/66 [p = 0.0005],

67/68 ; [p = 0.002], 69/70 [p = 0.004], 71/72 [p = 0.0008] respectively) and those spanning 75-80 min 75/76 [p=0.001], 77/78 [p = 0.003] 79/80 [p = 0.004] respectively) of the post-dose task period. No significant differences were found between resveratrol and placebo on day 28.

Mean total-, oxy- and deoxy-Hb levels for placebo and resveratrol, across day 1 and day 28, are shown in figure 2.

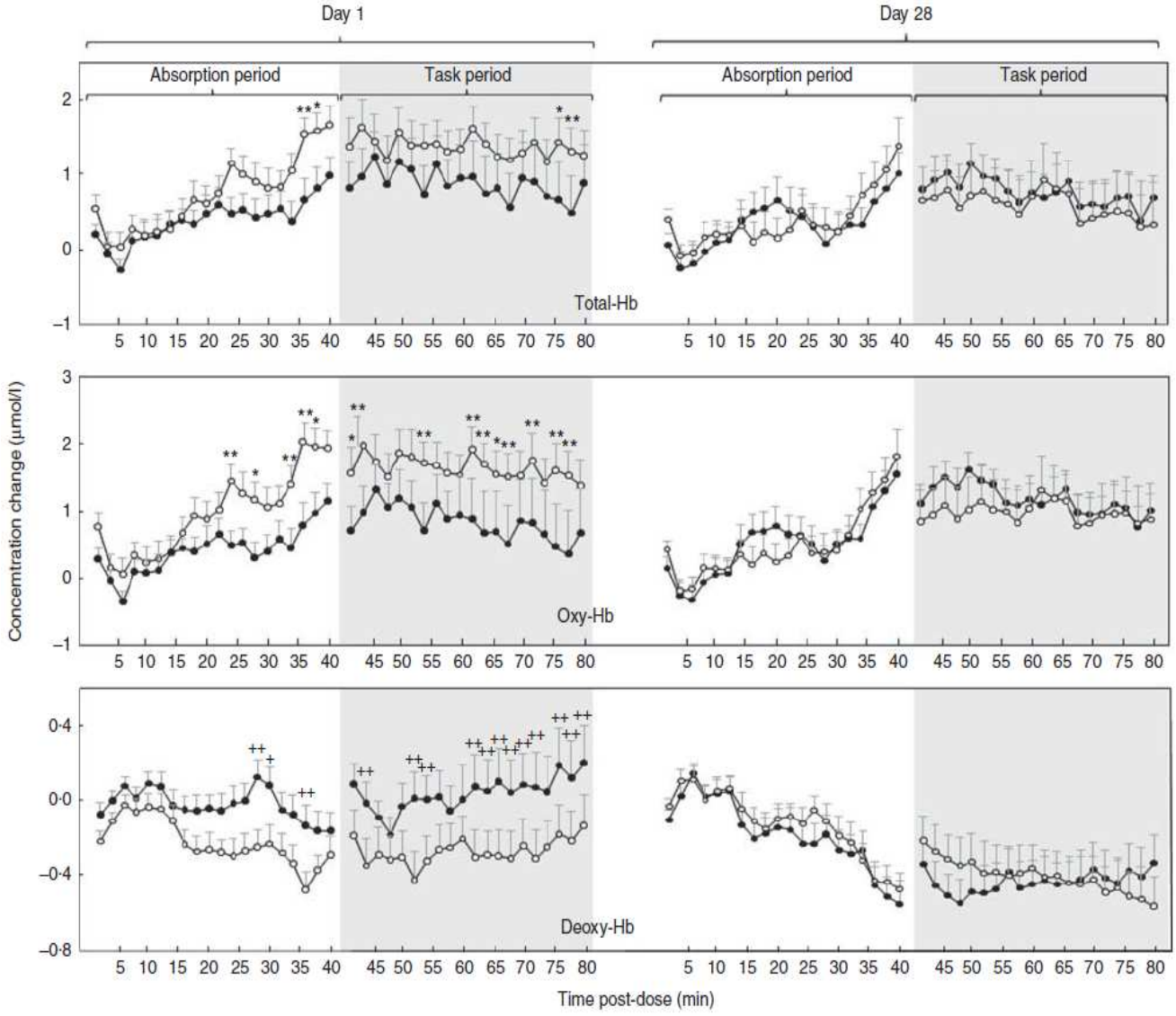

Figure 2. Concentration changes from baseline in levels of (top) total Hb (Total-Hb), (middle) oxygenated Hb (Oxy-Hb) and (bottom) deoxygenated Hb (Deoxy-Hb). Data averaged across two-min epochs during a 40-min absorption period and subsequent 40 min of cognitive task performance following placebo or 500 mg of resveratrol on day 1 and day 28 (n 46).● , Placebo;○ , 500 mg of resveratrol. Values are means, with standard errors represented by vertical bars. Significance planned comparisons (Bonferroni corrected) between resveratrol and placebo of data from each 2-min epoch: * P<0·05 and ** P <0·01.

TCD parameters

No significant acute chronic or gross chronic effects were observed with any of the 4 TCD parameters (Mean velocity; Peak systolic velocity; Diastolic velocity; and Pulsatility index).

Cognitive task performance

1. Pure chronic ANOVA

The results of the ANOVA on day 28 pre-dose data (converted to change from day 1 baseline) comparing performance between 500mg resveratrol and placebo, demonstrated a significant effect of treatment for the 3-Back task in terms of the % of correct responses ($F(1,40)= 8.60$, $p=.006$) with better performance in the resveratrol condition as compared to placebo.

2. Acute, chronic and superimposed ANOVA

The results of the treatment x repetition x day ANOVA are as follows. Note that, for brevity, only those significant main and/or interaction effects involving treatment are described here but see supplementary materials for all ANOVA F and P value tables.

7s incorrect: Analysis revealed a main effect of treatment ($F(1, 39)= 6.40$, $p=0.016$) (with the mean for number of serial 7s incorrect responses for placebo, overall, higher than the mean for 500mg resveratrol) and a day x repetition x treatment interaction ($F(3, 117)= .260$, $p=0.034$). Post-hoc comparisons (Bonferroni corrected) revealed a significant difference on day 1 at repetition 4 ($p=.005$) and trends for differences on day 1 at repetition 2 ($p= .073$) and on day 28 at repetition 3 ($p= .070$). The mean number of incorrect responses was lower in the 500mg resveratrol condition in all 3 cases.

17s correct: The ANOVA revealed an interaction between day x treatment x repetition ($F(3, 117)= 3.45$, $p=0.019$). Post-hoc comparisons revealed significant differences on day 28 at repetition 1 and repetition 3 (both $p=0.04$) with the mean number of serial 17s correct completions higher in the placebo condition in both cases.

17s incorrect: The ANOVA showed a main effect of treatment ($F(1, 39)= 5.79$, $p=0.021$) (with the mean number of 17s subtraction incorrect responses, overall, higher in the placebo condition as compared to 500mg resveratrol). An interaction between repetition x treatment ($F(3, 117)= 3.55$, $p=0.017$) was also observed. With regards the repetition x treatment interaction, post-hoc comparisons revealed only one significant comparison between treatments at the 4th repetition on day 28. Here the mean number of incorrect responses was higher ($p=0.003$) in the placebo condition.

General health

There were no significant treatment related differences on the General Health Questionnaire (GHQ) or its subcomponents.

Sleep

There were no significant treatment related differences on the Pittsburgh Sleep Quality Index (PSQI) or its subcomponents.

Mood

A significant treatment effect was observed for the 'fatigue' measure alone ($F(1, 52)= 9.37$, $p= 0.003$); derived from the Profile of Mood States (POMS) questionnaire. Further analysis with Bonferroni corrected post-hoc student t tests demonstrated that subjective ratings of fatigue were significantly lower for resveratrol on day 7 ($p=0.04$), day 21 ($p=0.013$) and day 28 ($p=0.001$). A move towards a trend was also evinced for day 14 ($p=.097$). See supplementary materials for average weekly ratings on POMS questionnaire and ANOVA F and P value tables.

Blood pressure

1. Pure chronic ANOVA

The results of the ANOVA on day 28 pre-dose BP measurements (converted to change from day 1 baseline) comparing readings between 500mg resveratrol and placebo, demonstrated only a significant effect for diastolic BP ($F(1, 28)= 5.86$, $p=0.022$) with levels higher in the resveratrol condition.

2. Acute, sub-chronic and superimposed ANOVA

No significant effects were observed for systolic BP or HR. For diastolic BP, a significant interaction between treatment x day was evinced ($F(1, 22)= 6.61p=0.017$) which revealed only 1 significant comparison, in the placebo condition, between day 1 and day 28, at the 40 minutes PD measurement ($p=0.46$). Here the mean was higher overall on day 28 compared to day 1.

See supplementary materials for BP values and ANOVA F and P value tables.

Plasma analysis (total metabolite levels)

A significant effect of time was observed ($F(1.35, 8.10)= 7.50$, $p= 0.02$) for levels of total resveratrol metabolites (the sum of Resveratrol 3-O-sulfate and Resveratrol 4'- and 3-O-glucuronides) with pairwise comparisons revealing that day 1 post-dose levels were higher than day 1 baseline ($p=0.023$), that day 28 pre-dose levels were higher than day 1 baseline ($p=0.033$) and that day 28 post-dose levels were higher than both day 1 baseline ($p=0.003$) and day 28 pre-dose levels

(p=0.005). All 3 metabolites followed this same pattern of significance and so, for brevity, only total metabolite levels are reported here.

No resveratrol (in any form) was found in baseline samples on day 1, indicating that all volunteers did not consume resveratrol containing products before the study. No aglycone resveratrol was quantifiable in plasma at any time-point, on either day. Resveratrol 3-O-sulfate was the predominant metabolite in all volunteers, contributing 73-77% of total metabolites. The 4'- and 3-O-glucuronide forms evinced roughly equal contributions to the remaining metabolites in circulation.

Mean plasma concentration values (µM) for resveratrol metabolites at baseline and post-dose (110 minutes after administration) on day 1 and, after daily 500mg consumption, on day 28 shown in figure 3.

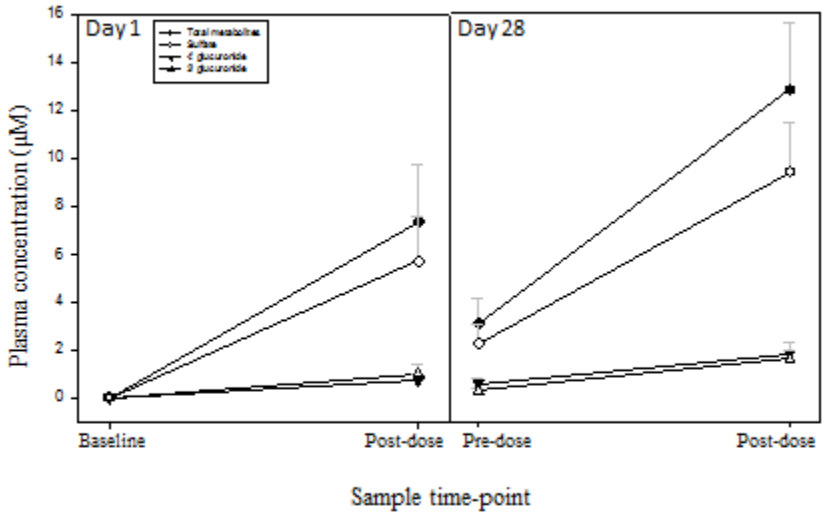

Figure 3. Mean plasma concentration (µM) values (±SEM) of resveratrol metabolites in plasma at baseline and post-dose (110mins post administration) on day 1 and day 28 (N=7). Graph displays mean plasma concentration (µM) values (with SEM error bars) of resveratrol metabolites in plasma at baseline and post-dose (110mins post administration) on day 1 and day 28, after 500mg *trans*-resveratrol, in 7 healthy, young adults. Significance on graph demonstrated for total metabolites, with * (P=<.05) and ** (p=<.01), although all 3 metabolites demonstrate the same pattern.

DISCUSSION

In summary, the results here show that whilst a single dose of 500mg *trans*-resveratrol can modulate CBF parameters in the frontal cortex in a pattern consistent with increased blood flow, supplementation for 28 days does not result in any clear improvements in cognitive function, despite an increase in plasma metabolites levels. However, there was evidence of significantly reduced fatigue and higher diastolic BP following extended supplementation. No modulation of subjective sleep quality, health or chronic CBF was observed.

The chronic 28 day dosing paradigm utilized in the current paper was designed to address the potential ineffectiveness of resveratrol at eliciting cognitive performance effects after acute, bolus supplementation[2, 33]. The hypothesis being that chronic consumption of resveratrol might increase exposure to resveratrol; a polyphenol with known low bioavailability following acute administration[9]. This increased exposure may be expected to enhance the biological activity of resveratrol; specifically, of importance here, those with direct and/or indirect effects on cognitive function. However, analysis demonstrated that the only cognitive task measure to evince a pure chronic effect (derived by the comparison of changes in performance between resveratrol and placebo between day 1 baseline and day 28 pre-dose) was N-Back % correct: i.e. after 28 days supplementation, participants in the 500mg resveratrol condition completed significantly more correct 3-Back responses before taking their day's treatment, as compared to placebo. No effects on this measure were observed following consumption of treatment on day 1 or day 28 nor were any effects observed on the other accuracy sub-measure assessed here. The results of acute and chronic/superimposed analysis revealed that, on day 28, participants in the resveratrol condition performed slower, achieving less correct responses on the serial 17 subtractions task. However, on day 1 and day 28, participants in the same condition also performed more accurately (less incorrect responses) on the serial 7 and serial 17 subtraction tasks. Whilst these results suggest a speed accuracy trade-off, closer inspection of these significant main effects highlights an inconsistent and difficult to interpret pattern, with the effects on the serial 7 task restricted to the 4th task battery repetition on day 1 only and the 1st, 3rd and 4th repetitions, on day 28, for the effects on the serial 17 subtraction task; where both higher and lower performance was seen in the resveratrol condition. Due to the lack of any clear pattern of results in both the acute and chronic effects of resveratrol on cognition here (and indeed the previous two studies assessing the effects of resveratrol on cognitive function), it is important to regard these results with caution. It may be that the relatively small sample here is masking a real effect, or a clearer effect, of resveratrol or it may be that a number of

type I errors have inflated expectations. Nevertheless, only a tightly controlled, crossover study with greater power would be able to address this issue.

The current study demonstrates that 500mg *trans*-resveratrol is able to augment the CBF response to cognitive task demands, relative to placebo, after acute, oral, administration to healthy human participants. This acute augmentation manifested in small, significantly higher levels of total-Hb, indicative of increased CBF, at the ends of the absorption- and post-dose task periods and a consistent pattern of significantly higher levels of oxy-Hb across some of the absorption- and post-dose task periods following the first dose of resveratrol on day 1. Levels of deoxy-Hb were also significantly lower in the resveratrol condition, as compared to placebo. This latter finding is directly opposite to that reported previously[2, 33] and is contrary to the hypothesis that resveratrol would facilitate increased oxygen extraction due to its reported effects on oxidative phosphorylation[36]. No clear reason for this anomalous finding can be offered at present but it may be notable that whilst the previous two aforementioned resveratrol/NIRS studies by this lab were crossover studies, the current is the first to utilize a between-subjects design and this may introduce an unanticipated degree of variability in CBF parameters. In contrast to day 1, the consumption of the resveratrol treatment on day 28 was not found to have an acute effect on any of the CBF parameters. As noted above, CW-NIRS generates concentration change, rather than quantitative data, and therefore only provides a measure of acute changes in haemodynamics during each discrete recording session. It therefore provides no direct measure of any changes that have taken place between recording sessions, in this case as a consequence of chronic resveratrol supplementation. The lack of an effect here may then reflect several distinct possibilities. It may, of course, reflect a simple attenuation of the acute effects seen following the first dose of resveratrol on day 1. However, it could equally reflect either the raised levels of resveratrol metabolites seen pre-treatment on day 28, which may have precluded a further acute effect of an additional dose on day 28; or it may indicate that a gross (undetected) change in CBF parameters had already taken place, attenuating the possibility of any additional acute effects of day 28's treatment.

In the current study, TCD was also incorporated to provide a measure of chronic CBF. This technique provides an absolute quantitative measure of CBF, (in this case as indexed by CBF velocity (CBFV) in the right middle cerebral artery) which was intended to elucidate any gross chronic changes in CBF as a consequence of resveratrol supplementation. No significant changes in CBFV were observed with TCD, suggesting a simple absence of modulation of CBF by resveratrol However, this interpretation should be tempered by several considerations. The first is that the recording period was much shorter (at 5 minutes) than for NIRS and it was undertaken entirely at

560 rest, with no data collected during the period of task-performance during which resveratrol has been
561 shown to have its most pronounced effects. Secondly, whilst the NIRS was used to measure local
562 changes in CBF in the upper layers of the frontal cortex during tasks which activate this brain area,
563 the right middle cerebral artery supplies the entire right side of the cortex. Given this, any
564 vasodilatory effects restricted to the locality of neural activity (in this case the prefrontal cortex)
565 may have been swamped in the gross blood flow. Potential reasons for a lack of significant CBFV
566 changes include the relatively short recording period with the TCD: 5 minutes, yielding only 2
567 measurements per minute, which may simply be to narrow a window to detect effects. The TCD
568 recording periods were also conducted during times of minimal cognitive demand (pre and post the
569 cognitive task periods) and, as such, metabolic substrate demands would have been less during
570 these periods and an increase in the hemodynamic response unnecessary. Ideally the TCD and
571 NIRS would both have been used to record concomitantly throughout the absorption and cognitive
572 task periods. Unfortunately, due to the physical constraints of the equipment utilized here, this was
573 not possible.

574 The current study does, however, report vascular effects of resveratrol in the periphery on day 28;
575 with the analysis of pure chronic effects (derived by comparing change from day 1 baseline BP
576 measurements between resveratrol and placebo to pre-treatment on day 28) demonstrating higher
577 diastolic BP in resveratrol-supplemented participants. No pre-treatment baseline differences in BP
578 readings, nor acute effects of treatment within day 1 or day 28 were observed. This finding is
579 intuitively unexpected as resveratrol has previously been shown to be a vasodilator[6, 37]; a
580 phenomenon associated with lowered BP. Whether resveratrol can act as a vasoconstrictor is, at
581 present, unknown but it may be noteworthy that structurally similar polyphenols, such as the tea
582 polyphenol epigallocatechin-3-gallate (EGCG), can act both as both vasodilators and
583 vasoconstrictors depending on dose and the time of assessment[38]. EGCG has also been
584 investigated with regards its cognitive and CBF effects in humans, with a single dose of 135mg,
585 leading to a significant reduction in CBF as compared to placebo; which might indeed be suggestive
586 of vasoconstriction.

587 No significant differences between treatments, or within-treatment changes, were observed with
588 subjective perceptions of general health (as assessed by the GHQ) or sleep (as assessed by the
589 PSQI). With regards subjective perceptions of mood, the only variable on the POMS questionnaire
590 which evinced any significant difference was ‘fatigue’ which remained significantly lower across
591 the entire 28 day period in the resveratrol condition, as compared to placebo. Little research exists
592 regarding the effects of polyphenols on mood but this anti-fatigue effect may find an explanation in

593 *in vitro* and animal work which reports the ability of resveratrol to inhibit Monoamine Oxidase-A
594 and B (MAO-A/B) activity. This inhibition was reported to lead to an increase in monoamine
595 neurotransmitter concentrations, namely 5-hydroxytryptophan (5-HT), noradrenaline and dopamine,
596 with a concomitant improvement in mood; similar to that seen with imipramine and fluoxetine, in
597 mice[18]. Interestingly quercetin, another red wine polyphenol, also shows anti-fatigue activity
598 through increased energy expenditure and endurance capacity in mice[39, 40 respectively] and power
599 output in elite male cyclists when part of a cocktail of supplemented compounds[41]. Mechanisms
600 include increased blood flow; due to vasorelaxation[42], and oxygenation; with Davis et al.[40] also
601 reporting SIRT-mediated increases in mitochondrial gene expression in brain and skeletal muscles.
602 Both mechanisms are shared with resveratrol[36, 42] and could explain the increased energy levels
603 seen here. It is worth noting here that, whilst there was no statistically significant difference in
604 baseline (pre-dose on day 1) levels of fatigue between resveratrol and placebo participants, the
605 baseline values were nevertheless numerically higher in the former group (8.04 compared to 5.54
606 respectively) which might suggest that this effect represents a return to normal levels for the
607 resveratrol group following an unusually high baseline.

608 Analysis of the plasma samples, taken from a sub-sample of 7 participants from the resveratrol
609 condition on day 1, demonstrated increases in acute resveratrol metabolite levels post-dose very
610 similar to those seen in a previous study conducted by this lab[2]. Pre-dose levels of metabolites on
611 day 28 were also significantly higher than those seen pre-dose on day 1, suggesting that chronic
612 consumption results in an accumulation of resveratrol metabolites in plasma. They subsequently
613 increased following day 28's treatment, and again ended at a significantly higher level than post-
614 dose on day 1. Pre- and post-dose levels of resveratrol on day 28 were significantly higher than
615 baseline levels on day 1 and, within day 28, post-dose levels were significantly higher than pre-dose
616 levels. Taken together, these findings suggest (hence their presence prior to treatment
617 administration on day 28), and that this may amplify the increase following acute administration
618 (hence numerically higher levels at day 28 post-dose compared to day 1 post-dose). That the day 1
619 baseline mean levels were 0 does render this comparison, statistically, problematic. However,
620 disregarding statistical significance, the fact that metabolites were present on day 28 at all
621 (considering that levels were 0 at baseline on day 1) is indicative that an increase in plasma levels of
622 resveratrol had taken place. This novel finding of accumulating levels of resveratrol metabolites as
623 a consequence of chronic administration certainly warrants further investigation with larger
624 samples, as previous acute dose research does not suggest that plasma metabolites should still be
625 present beyond 24hrs[9], or certainly not at the levels seen here at pre-dose on day 28[10]. It may be

possible that these effects are the result of some other, unknown factor/s; for instance the consumption by participants of more resveratrol containing products or an additional resveratrol capsule prior to attending the laboratory on day 28. However, this seems unlikely, and is argued against by the participants' treatment diaries and a capsule count.

The methodology of the current study had a number of strengths and limitations. The nature of the paradigm; namely the timeframe involved and the use of equipment which dictates individual testing (i.e. the NIRS and TCD), necessarily means that the sample size is somewhat restricted for outcome measures like cognitive performance which ideally require a larger sample than the physiological measures. In this study the issue was exacerbated by the loss of a number of sets of data (due largely to an equipment failure) which reduced the number of cognitive performance data sets. This renders interpretation of the cognitive data more difficult, but an argued strength of this paper is the caution with which the authors have regarded such data. Another limitation relates to the equipment utilized here to measure CBF. As noted above CW-NIRS only generates acute concentration change data, and therefore the question that it was used to address on day 28 of the current study was: "Are the acute haemodynamic effects of the single dose of resveratrol taken on day 28 the same, or different, to those seen following the first dose taken on day 1". The results showed that there were no acute effects on day 28, so they were different. However, the difficulty in interpreting this finding further is that this could reflect an attenuation of the acute effects over time, but it could equally be the result either of the raised levels of resveratrol metabolites already seen prior to taking the day 28 treatment, or indeed unmeasured chronic effects on CBF. To address the last of these points TCD was incorporated as a measure of chronic changes of absolute CBFV, but this measure showed no effect- although again this could be due to methodological issues (including measuring at rest, rather than during task performance, and the diffuse rather than local nature of the measurement). It would therefore be advantageous to revisit the question of the chronic effects of resveratrol on CBF using the more recently introduced 'quantitative' NIRS, which, as the name suggests, generates quantitative, rather than concentration change data. In terms of strengths, the current paper incorporated a range of methodologies in order to answer the, hitherto unaddressed question, as to whether resveratrol can engender chronic cognitive effects. This is also the first paper to show that repeated consumption of resveratrol can lead to cumulative plasma levels at a dose which is recommended by many over-the-counter resveratrol products.

In conclusion, the current study reports that chronic, 28 day supplementation of 500mg *trans*-resveratrol results in significantly reduced fatigue and higher diastolic BP, but does not modulate sleep, health or chronic CBF. The single, chronic, cognitive effect evinced by resveratrol and the

confusing pattern of acute effects, should be treated with caution. This study is the first to suggest that chronic resveratrol consumption could result in cumulative plasma levels in healthy humans after oral administration.

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