Effects of resveratrol alone or in combination with piperine on cerebral blood flow parameters and cognitive performance in humans: a randomised, double-blind, placebo-controlled, crossover investigation.

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ABSTRACT

Previous research has shown that resveratrol is able to increase cerebral blood flow (CBF) in the absence of improved cognitive performance, in healthy, young humans during cognitively demanding tasks. This lack of cognitive effects may be due to low bioavailability and, in turn, reduced bioefficacy of resveratrol *in vivo*. Piperine is able to alter polyphenol pharmacokinetics but previous studies have not investigated whether this affects the efficacy of the target compound. Taken together, the objective here was to ascertain if piperine co-supplementation might affect the bioavailability and efficacy of resveratrol with regards cognition and CBF. This investigation utilized a randomised, double-blind, placebo-controlled, within subjects design, where 23 adults received placebo, *trans*-resveratrol (250mg), and *trans*-resveratrol with 20mg piperine, on separate days at least a week apart. After a 40min rest/absorption, participants performed a selection of cognitive tasks and CBF was assessed throughout, in the frontal cortex, using Near-Infrared Spectroscopy (NIRS). The presence of resveratrol and its conjugates in plasma were confirmed by LC-MS following the same doses in a separate cohort (N=6). The results indicated that when co-supplemented, piperine and resveratrol significantly augmented CBF during task performance in comparison to placebo and resveratrol alone. Cognitive function, mood and blood pressure were not affected. Plasma levels of resveratrol and its metabolites were not significantly different between treatments which indicates that piperine co-supplementation enhances the bioefficacy of resveratrol with regards CBF effects, but not cognitive performance, and does this without altering bioavailability.

INTRODUCTION

Resveratrol (3, 4', 5 trihydroxystilbene) is a polyphenolic secondary metabolite produced within plants in response to a range of environmental stressors [1]. Resveratrol ingestion has also been shown to have protective effects in animals and humans. Of direct relevance here, these effects include a protection of cognitive function/reversal of cognitive deficits in animal models following supplementation [2] which may, in large part, be due to the cerebral blood flow (CBF) effects evinced by resveratrol [3]. These CBF effects are likely to be mediated by the ability of resveratrol to modulate nitric oxide (NO) synthesis [4], with oral intervention shown to enhance endothelium-dependent relaxation in rats [5, 6], and improve flow- mediated dilatation in overweight/obese humans [7]. An increase in blood-borne neural metabolic substrates such as oxygen [8] and glucose [9] is reported to enhance aspects of cognitive performance in healthy, young humans. Taken together, it could be hypothesized that an acute increase in CBF, augmenting the delivery of metabolic substrates, might also beneficially affect cognitive performance.

A recent study from this laboratory demonstrated a dose-related increase in pre-frontal cortex CBF during cognitively demanding tasks in healthy, young adults. This effect was consistent across all time points for 500mg, but failed to reach significance for 250mg of resveratrol. The increase in CBF did not facilitate improved cognitive task performance [10]. It was argued that this may be due to low bioavailability of resveratrol.

The pepper derived alkaloid piperine has been observed to be a potent enhancer of the bioavailability of numerous compounds, including polyphenols, *in vivo*; for instance, epigallocatechin-3-gallate (EGCG) in rodents [11], curcumin in rats and humans [12], and beta-carotene following 14-days co-supplementation in humans [13]. With regards resveratrol, piperine co-supplementation (10mg/kg) is reported to evince a 1544% enhancement of maximum serum resveratrol levels (compared to 100mg/kg resveratrol alone) and increase exposure (AUC) by 229% in mice [14]. Potential mechanisms for these phenomena include inhibition of enzymes responsible for metabolising polyphenols [14-16]; enhancement of metabolism via thermogenic effects [13]; and/or competing for membrane efflux pumps in the body and brain: a phenomena seen when plant derived compounds are co-administered, for example polyphenols [17]. These studies, however, did not investigate whether increased bioavailability led to increased bioefficacy of the target compound.

The current randomised, double-blind, placebo-controlled, cross-over study therefore investigated the effects of 250mg resveratrol when administered alone, and when co-supplemented with 20mg of

piperine. The rationale for utilizing 250mg resveratrol here is based on the previous ineffectiveness of this dose in modulating CBF and the expectation that this will be augmented by the actions of piperine. The aim was to ascertain if piperine is capable of enhancing the bioefficacy of resveratrol with regards CBF and cognitive performance in healthy adults. Blood plasma levels of resveratrol were collected to investigate whether bioavailability correlated with bioefficacy.

EXPERIMENTAL METHODS

Participants (*CBF and cognitive performance assessment):*

23 healthy adults (4 males, 19 females, mean age 21yrs, range 19-34yrs, SD 3.2yrs, all right handed) took part in all three arms of the cross-over study. The data from 1 participant was excluded from analysis due to data catchment errors. All participants attended the laboratory after a 12hr overnight fast and reported to meet the inclusion criteria: i.e. to be in good health and free from social drugs, alcohol, prescription medication, herbal extracts/food supplements, relevant food allergies, intolerances and digestive problems. A fasted state was considered to be most appropriate due to the individual differences involved with breakfast consumption and the unknowns involved with the absorption of resveratrol together with food. Whilst food deprivation has been reported to deleteriously affect cognitive function previously in children [18, 19] actually more recent research with athletes during Ramadan is more ambiguous [20] and a well-controlled study of healthy, young adults finds no detrimental effects of fasting on cognitive performance [21]. All participants were non-smokers and did not consume excessive amounts of caffeine (>6 cups of coffee or equivalent/d). In addition, participants who had suffered a head injury, neurological disorder or neuro-developmental disorder were excluded from participation, as were those who had uncorrected sight problems, were pregnant or seeking to become so.

Participants (Bioavailability analysis):

6 healthy (mean BMI 24.2, range 21.7-27.2, SD 2.38) male adults (mean age 25.8yrs, range 23-29yrs) took part in the bioavailability assessment. Inclusion/exclusion criteria were as per the CBF and cognitive performance aspect of the study.

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the department of Psychology ethics committee at Northumbria University. Written informed consent was obtained from all subjects.

Treatments:

During the three study visits participants received three single-dose treatments in an order dictated by random allocation to a counterbalancing (Latin Square) order. The three treatments comprised two capsules; each combination delivering either an inert placebo, 250mg of *trans*-resveratrol or 250mg of *trans*-resveratrol plus 20mg piperine. The treatments were administered in identical size 0 vegetable capsules, which were prepared by the lead researcher 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.

Near-Infrared spectroscopy (NIRS):

Relative changes in the absorption of near-infrared light were measured at a time resolution of 10Hz using a 12-channel Oxymon system (Artinis Medical Systems B.V.). The system emitted two nominal wavelengths of light (~765- and 855nm) with an emitter/optode separation distance of 4cm. The differential pathlength factor was adjusted according to the age of the participant. Relative concentration changes in oxy-Hb, deoxy-Hb and total-Hb were calculated by means of a modified Beer-Lambert law [(22)] using the proprietorial software. Given the extended recording period and the investigational aims, a simple two emitter/optode pair configuration was utilised (i.e. 2 channels). The emitter/optode pairs were positioned over the left and right frontal cortex using a standard optode holder headband, which separated the pairs from each other by 4cm. Each pair therefore collected data from an area of prefrontal cortex that included the areas corresponding to the International 10-20 system Fp1 and Fp2 EEG positions. The NIRS data output was time stamped at the start of each task segment to assure that data corresponded to the relevant epoch of task performance.

Cognitive tasks:

In order to maximise cerebral activity-induced modulation of blood flow, a pilot study was initially carried out with a separate cohort of 15 participants (3 male, 12 female, mean age 21.6yrs, all right handed) to ascertain the most 'mentally demanding' and 'difficult' tasks from a battery of 11. (Data not reported.) The 5 tasks utilized here were all subjectively rated as both the most 'demanding' and most 'difficult' and have all previously been shown to activate the frontal cortex in fMRI studies [(23-25)]. The computerised battery of cognitive tasks were delivered using the Computerised Assessment of Mental Performance System (COMPASS) 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 task and RVIP are described in detail in [10].

N-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 includes sufficient stimuli (letters) to last for at least 2 minutes although this is dependent on speed (i.e. slower reaction times will result in a lengthier task) and is scored for accuracy and reaction time.

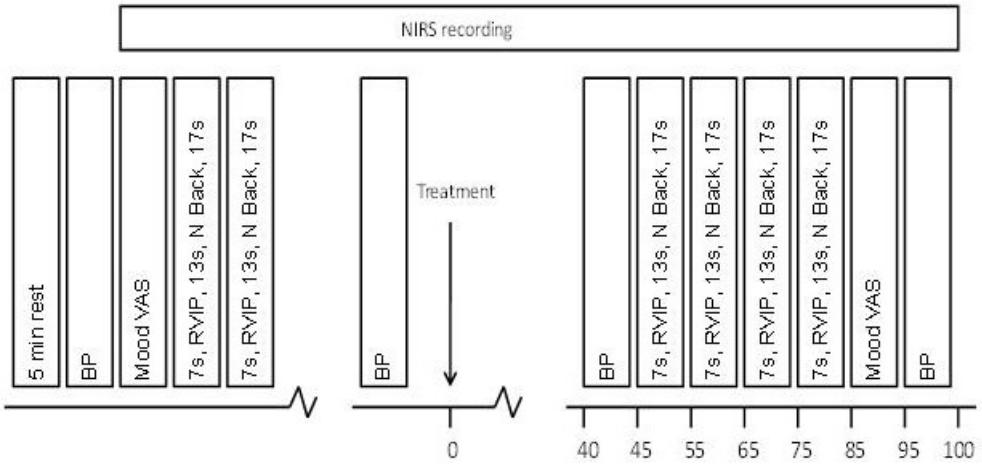
Mood Visual Analogue Scales (Mood VAS): Participants were required to rate how 'relaxed', 'alert', 'jittery', 'tired', 'tense' and 'mentally fatigued' they felt by placing a cross with the mouse and cursor on a 100mm on-screen line between the descriptors 'not at all' and 'extremely'. They also rated their 'overall mood' on a scale anchored by 'very poor' to 'very good' and their levels of 'headache' between 'not at all' and 'extremely'. The VAS were scored as % along the line denoting more of the relevant adjective.

Procedure (*CBF and cognitive performance assessment):*

Each participant was required to attend the laboratory on 4 occasions. The first of these was an initial screening/training visit during which participants provided written informed consent, were screened with regards the study inclusion/exclusion criteria, briefed with regards compliance requirements and given training in completing the cognitive tasks. This visit was followed within 14 days by the first of 3 active study mornings.

On each of the 3 active study mornings, which were conducted 2-14 days apart, participants attended the laboratory at 8:30am in a fasted state and provided confirmation of continued compliance with regards the inclusion/exclusion requirements. After a 5 minute seated resting period a blood pressure reading was taken after which the NIRS headband was fitted. Participants then completed a series of mood VAS and 2 repetitions of baseline cognitive tasks in the following order: Serial 7s, RVIP, Serial 13s, N-Back, and Serial 17s. Participants then rested for 10 minutes and provided a 2nd blood pressure reading. Treatment was then administered after which participants sat quietly, watching one of a selection of non-arousing DVDs, for a 40 minute

189 'absorption' period. Following this time a 3rd blood pressure reading was taken after which
190 participants completed 4 repetitions of the aforementioned tasks in the same order and duration.
191 After the post dose tasks were completed the same mood VAS were presented and the 4th and final
192 blood pressure reading was taken. NIRS data was captured throughout. The timeline and running
193 order of the testing session are shown in Figure 1.



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195 **Figure 1. Testing session timeline.** Upon arrival participants rested for 5-min before the 1st blood pressure
196 reading was taken. The NIRS headband was then fitted. Mood visual analogue scales (VAS) and 2
197 repetitions of baseline cognitive tasks were completed and followed by a 10-min rest. The 2nd blood pressure
198 reading was then taken and treatment was administered. After a 40-min absorption period the 3rd blood
199 pressure reading was taken. 4 repetitions of the cognitively demanding tasks were then completed, followed
200 by mood VAS and the 4th and final blood pressure.

201

202 *Procedure (bioavailability assessment):*

203 On each study morning participants attended the laboratory at 8.30am. Venous blood samples were
204 collected using 4.7ml monovettes (containing lithium heparin) before the day's treatment was
205 consumed and then 45-, 90- and 120 minutes after consuming intervention. Samples were
206 centrifuged at 2500rpm for 15min at 20°C to yield plasma, which was then stored at -80°C until
207 analysis.

208

209 *Preparation of Samples:*

210 Samples were handled in low light conditions to reduce the scope for isomerisation. Plasma was
211 defrosted at room temperature immediately before extraction, vortexed then sonicated for 5min. A
212 200µL aliquot was mixed with 900µL of HPLC grade ethanol plus 0.1% formic acid (v/v), along
213 with 100µL of naringenin internal standard (IS1; Extrasynthese, France) in ethanol (500ng/ml).
214 Samples were vortexed, sonicated and then separated via micro-centrifugation at 17k R.C.F. for

10min. The supernatant was removed and placed in an amber 1.5ml centrifuge tube (Eppendorf, UK). The pellet was re-extracted with 1.2ml of 83% aqueous ethanol (v/v) following the same protocol. Both extracts were evaporated to dryness under vacuum using a centrifugal evaporator (EZ2+, Genevac, UK), and frozen at -20^{o}C. On the day of analysis, a 70µL portion of ethanol was added to the secondary extract, which was vortexed and sonicated. A 50µL aliquot of this solution was then added to the primary extract, which following vortexing and sonication was mixed with 50µL taxifolin (IS2 at 2µg/ml; Extrasynthese, France) in 0.2% ascorbic acid solution. This solution was vortexed, separated by centrifugation and the supernatant placed in an amber vial and analyzed via LC-MS. Extractions were made in duplicate for each time point. To test extraction efficiency of this method, blank plasma was spiked with standards at 50nM, 500nM, 5µM and 10µM concentrations. Across this range, the average extraction efficiencies for *trans*-resveratrol (Cayman Chemicals, USA), the -3-*O*-sulfate, 4-*O*'-glucuronide and 3-*O*-glucuronide (Bertin Pharma, France) were 74%, 72%, 52% and 55%, respectively. IS1 and IS2 were extracted consistently at 82% and 100%, respectively.

LC-MS Analysis:

Analysis was conducted using a Shimadzu LC2010CHT HPLC, consisting of an integrated quaternary pump, degasser, chilled autosampler (8^{o}C), and column oven (30^{o}C), connected to an LCMS2020 single quadrupole mass spectrometer. A 10µL sample aliquot was separated on an XDB–C18 1.8µ, 4.6 x 50mm column (Agilent, UK), running a binary gradient of LCMS grade water vs. acetonitrile, both containing 0.1% formic acid (v/v), running at 0.5ml/min. The gradient started at 5% acetonitrile, and moved to 10% at 5min, 40% at 20min and 90% at 25min. Following 4min of washing, the column returned to 5% acetonitrile at 30min and was re-equilibrated over 3min. The MS ran with an interface temperature set to 350^{o}C, using nebuliser and drying gas flow rates of 1.5- and 15L/min, respectively. The analysis was performed in negative SIM mode, following m/z of 403 (glucuronides), 307 (sulfates) 271 (naringenin IS1), 303 (taxifolin IS2) and 227 (aglycone resveratrol). A persistent formate adduct of aglycone resveratrol (m/z 273) was also followed as a qualifying ion. The limit of quantification (LOQ) for glucurnoides was 16nM, 22nM for sulfates, and 145nM and 290nM for *cis*- and *trans*-aglycone resveratrol respectively. Peak areas were normalized to IS2 for quantification, whilst IS1 was used to judge individual sample extraction. The retention times of *cis*-isomer resveratrol conjugates were identified by subjecting commercially available *trans*-isomers (10 µg/ml in 50% aqueous ethanol, plus 0.1% ascorbic and

247 0.05% formic acids) to ultraviolet light (254 nm) for 4 hr. *Cis*-isomer resveratrol conjugates were
248 quantified as *trans*- isomer equivalents, and then summed with the corresponding *trans*- isomers.
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250 *Statistics:*
251 The analyses of plasma data was conducted with SPSS 16.0 for Windows (SPSS Inc, Chicago, IL)
252 utilizing within subjects analysis of variance (ANOVA) (treatment x time) for each metabolite and
253 paired samples t-tests to compare AUC, Cmax and Tmax, between the 2 treatments, for each
254 metabolite.

255 NIRS data was analysed with Minitab 16 for Windows (Minitab Inc, State College, PA). For each
256 variable (oxy-Hb, deoxy-Hb and total-Hb), data was converted to 'change from baseline'
257 (calculated from a 10 minute pre-treatment resting period) and averaged across 2 minute epochs
258 during the 40 minute 'rest/absorption' and 40 minute cognitive task performance period. Analysis
259 was based on an average of the 2 NIRS channels to give a measure of cerebral hemodynamics
260 across the prefrontal cortex as a whole; in line with [10].

261 The primary analysis of the averaged NIRS data was conducted by within-subjects ANOVA
262 (treatment x 2 min epoch) with *a priori* planned comparisons of data from each epoch being made
263 between placebo and each of the resveratrol treatment groups (250mg resveratrol, 250mg
264 resveratrol with 20mg piperine) using t-tests calculated with the Mean Squares Error from the
265 ANOVA [26]. In order to protect against the possibility of type 1 errors, planned comparisons are
266 only reported if they evinced a consistent pattern of significant effects across the analysis period.

267 Task performance data (also analysed with SPSS 16.0) was analysed as change from pre-dose
268 baseline for each individual task (Serial 7s, RVIP, Serial 13s, 3-back and Serial 17s) by within-
269 subjects ANOVA (treatment x repetition), with planned comparisons for data from each repetition
270 as described above.

271 A power calculation conducted using G Power [27] suggested that a sample size of 24 would be
272 adequate to have greater than an 80% chance of detecting the medium effect sizes demonstrated in
273 previous research assessing the effect of resveratrol on NIRS parameters [10].
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RESULTS

NIRS parameters

Total haemoglobin (total-Hb)

A significant interaction was found between post-dose epoch and treatment ($P < 0.01$) on the ANOVA of total-Hb data. Planned comparisons showed that, compared to placebo, the 250mg resveratrol treatment failed to elicit any modulation of total-Hb levels. However, following 250mg resveratrol combined with 20mg piperine, whilst there were no significant effects during the absorption period, levels of total haemoglobin were significantly raised for all task performance epochs (apart from 45, 51 and 79 minutes). Time-points 41, 49 and 61 were all significant at the .05 level and the remainder at .01.

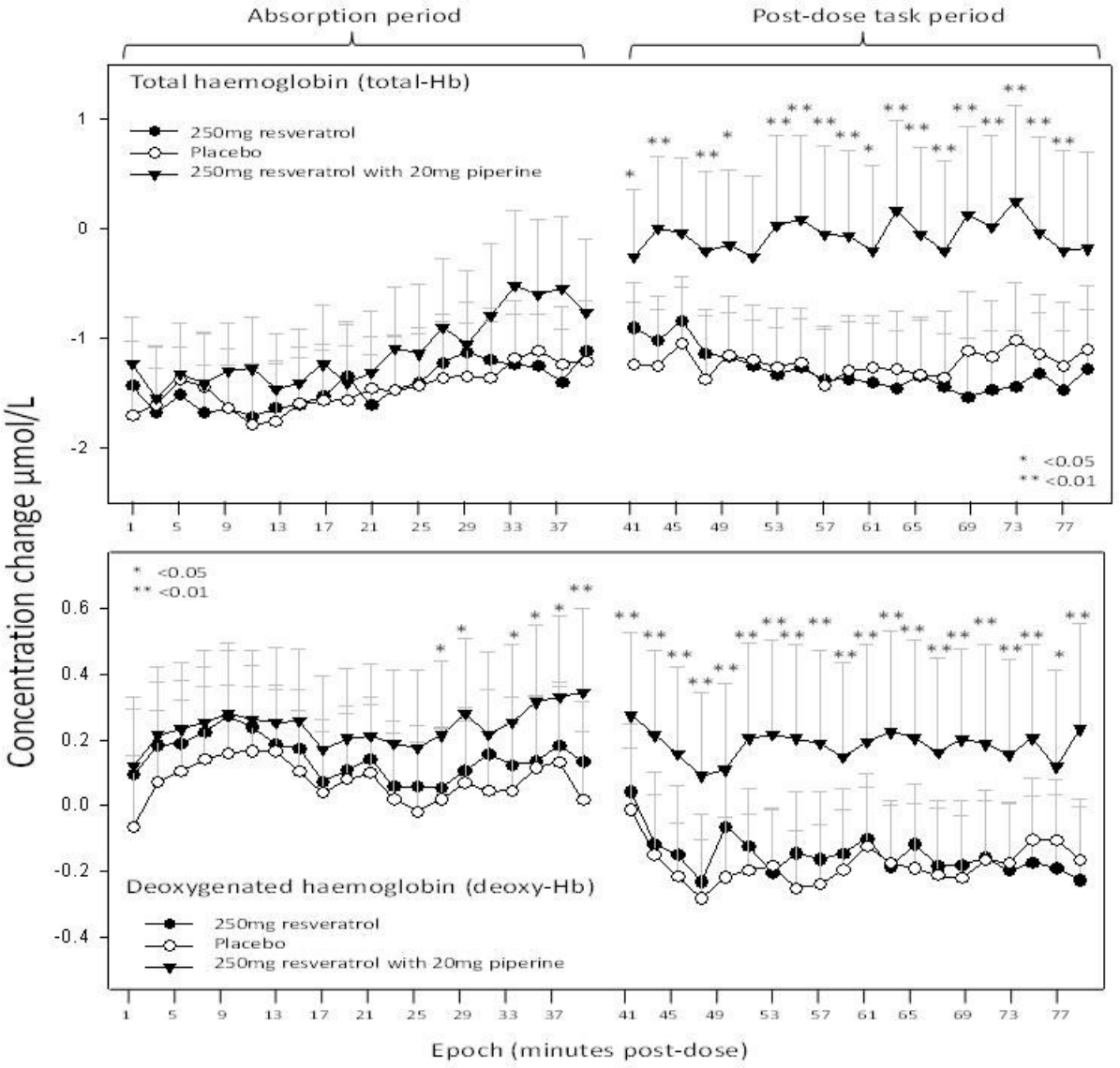
Oxygenated haemoglobin (oxy-Hb)

ANOVA showed that there was a significant interaction between the post-dose epoch and treatment ($P < 0.05$). The pattern was similar to that seen with regards total-Hb, with no modulation seen following 250mg resveratrol, but significantly raised concentrations of oxy-Hb seen following 250mg resveratrol with 20mg piperine (all epochs $P < 0.01$, except 45, 49 and 51 which were $P < 0.05$ and 79 which just failed to reach significance).

Deoxygenated haemoglobin (deoxy-Hb)

ANOVA showed that there was no significant main effect or interaction between time and treatment with regards deoxy-Hb. Planned comparisons, however, demonstrated a consistent pattern of significant effects which began to emerge during the end of the absorption phase and continued throughout the post-dose task period. After the 250mg resveratrol with 20mg piperine dose, levels of deoxy-Hb were significantly raised in comparison to placebo (during the absorption period epochs 27, 29, 33, 35 and 37 <0.05 and 39 <0.01; during post-dose task period all epochs <0.01 apart from 77 which was <0.05).

The mean data (± SEM) and the results of the planned comparisons for total-, and deoxy-Hb are represented in Figure 2.



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312 **Figure 2. Hemodynamic effects of 250mg resveratrol alone, and when co-supplemented with 20mg**
313 **piperine, in healthy, young humans.** Mean (±SEM), change from baseline, concentration changes in total
314 levels of haemoglobin (total-Hb) and deoxygenated haemoglobin (deoxy-Hb) during a 40-min absorption
315 period and subsequent 40-min of cognitive task performance following placebo (○), 250mg *trans*-resveratrol
316 (●), and 250mg *trans*-resveratrol with 20mg piperine (▼). The study followed a cross-over design (n= 23
317 per condition). Data are averaged across 2 minute epochs. *A priori* planned comparisons comparing data
318 from each resveratrol group to placebo for each epoch were carried out with t-tests incorporating Mean
319 Squares Error from an initial ANOVA. Significance on the planned comparisons is indicated by * (P< 0.05)
320 and ** (P< 0.01).

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Cognitive task performance and mood

There were no significant, treatment related differences on any cognitive or mood measures. The raw baseline and change from baseline mean task scores and mood ratings can be found in tables 1 and 2 respectively.

Table 1. Effects of resveratrol on cognitive performance. Mean values with their standard errors. N= 23. T= treatment; R= repetition; RVIP= rapid visual information processing. *=P<0·05, **=P<0·01.

Measure	Treatment condition	Task battery repetition				
		Baseline	1	2	3	4
7s Total (Number)	250mg resveratrol	30.72 *(2.66)*	1.54 *(0.82)*	1.72 *(0.74)*	1.89 *(0.71)*	1.59 *(1.06)*
	250mg resveratrol with 20mg Piperine	30.52 *(2.58)*	1.61 *(0.65)*	0.65 *(0.72)*	1.70 *(1.18)*	1.70 *(1.07)*
	Placebo	30.76 *(2.00)*	2.28 *(0.91)*	1.20 *(0.96)*	0.94 *(1.08)*	1.11 *(1.06)*
7s Correct (Number)	250mg resveratrol	28.85 *(2.75)*	1.20 *(1.02)*	1.98 *(0.94)*	1.54 *(0.83)*	0.80 *(1.16)*
	250mg resveratrol with 20mg Piperine	28.83 *(2.59)*	1.52 *(0.85)*	-0.04 *(0.94)*	0.39 *(1.25)*	0.57 *(1.13)*
	Placebo	28.85 *(2.04)*	1.94 *(1.12)*	0.89 *(1.11)*	0.11 *(1.43)*	0.98 *(1.29)*
7s Incorrect (Number)	250mg resveratrol	1.87 *(0.30)*	0.35 *(0.51)*	-0.26 *(0.37)*	0.35 *(0.38)*	0.70 *(0.55)*
	250mg resveratrol with 20mg Piperine	1.67 *(0.23)*	0.11 *(0.39)*	0.67 *(0.38)*	1.33 *(0.52)*	1.07 *(0.49)*
	Placebo	1.91 *(0.26)*	0.30 *(0.52)*	0.30 *(0.47)*	0.78 *(0.60)*	0.13 *(0.46)*
13s Total (Number)	250mg resveratrol	24.28 *(2.24)*	0.80 *(0.84)*	1.24 *(0.79)*	0.85 *(0.72)*	-0.02 *(0.78)*
	250mg resveratrol with 20mg Piperine	24.37 *(2.09)*	1.50 *(0.53)*	0.41 *(0.71)*	1.46 *(0.79)*	1.80 *(0.69)*
	Placebo	24.22 *(1.50)*	2.17 *(0.77)*	1.74 *(0.86)*	2.00 *(0.90)*	1.52 *(0.73)*
13s Correct (Number)	250mg resveratrol	22.22 *(2.25)*	0.70 *(0.88)*	-0.78 *(0.90)*	0.22 *(0.87)*	-1.17 *(0.98)*
	250mg resveratrol with 20mg Piperine	22.46 *(2.17)*	1.33 *(0.75)*	-1.15 *(1.26)*	-0.11 *(1.25)*	1.07 *(0.87)*
	Placebo	21.83 *(1.60)*	3.26 *(0.83)*	0.78 *(1.41)*	1.17 *(1.20)*	1.09 *(1.03)*
13s Incorrect (Number)	250mg resveratrol	2.04 *(0.23)*	0.13 *(0.40)*	2.04 *(1.00)*	0.65 *(0.51)*	1.17 *(0.47)*
	250mg resveratrol with 20mg Piperine	1.89 *(0.36)*	0.11 *(0.44)*	1.59 *(0.94)*	1.59 *(0.63)*	0.76 *(0.73)*
	Placebo	2.39 *(0.36)*	-1.09 *(0.33)*	0.96 *(0.84)*	0.78 *(0.53)*	0.44 *(0.58)*
17s Total (Number)	250mg resveratrol	19.50 (1.68)	1.54 *(0.58)*	1.50 *(0.56)*	2.59 *(0.55)*	2.67 *(0.56)*
	250mg resveratrol with 20mg Piperine	19.98 *(1.61)*	0.67 *(0.72)*	0.72 *(0.72)*	1.41 *(0.70)*	2.63 *(0.64)*
	Placebo	19.39 *(1.28)*	1.39 *(0.60)*	0.91 *(0.55)*	1.57 *(0.58)*	1.83 *(0.51)*

17s Correct (Number)	250mg resveratrol	17.22 *(1.68)*	1.39 *(0.71)*	1.48 *(0.81)*	2.35 *(0.75)*	1.09 *(1.13)*
	250mg resveratrol with 20mg Piperine	17.78 *(1.61)*	0.39 *(0.63)*	0.44 *(0.86)*	0.87 *(0.90)*	2.13 *(0.76)*
	Placebo	16.80 *(1.29)*	1.72 *(0.62)*	1.37 *(0.68)*	1.15 *(0.95)*	1.89 *(0.59)*
17s Incorrect (Number)	250mg resveratrol	2.28 *(0.28)*	0.15 *(0.41)*	0.02 *(0.47)*	0.24 *(0.52)*	1.54 *(1.09)*
	250mg resveratrol with 20mg Piperine	2.17 *(0.29)*	0.30 *(0.42)*	0.30 *(0.50)*	0.57 *(0.42)*	0.52 *(0.37)*
	Placebo	2.57 *(0.27)*	-0.30 *(0.37)*	-0.44 *(0.45)*	0.44 *(0.67)*	-0.04 *(0.36)*
N-Back Accuracy (%)	250mg resveratrol	93.38 *(1.17)*	-0.34 *(0.97)*	-1.02 *(1.05)*	-0.92 *(1.08)*	-0.05 *(1.00)*
	250mg resveratrol with 20mg Piperine	94.40 *(0.91)*	-2.03 *(1.02)*	-1.84 *(1.09)*	-0.29 *(0.89)*	-1.45 *(1.27)*
	Placebo	94.40 *(0.74)*	-1.26 *(1.08)*	-1.55 *(0.92)*	-2.61 *(1.13)*	01.45 *(0.93)*
N-Back Reaction Time (msec)	250mg resveratrol	1540.45 *(145.80)*	-291.04 *(48.75)*	-345.87 *(53.98)*	-312.95 *(52.58)*	-398.24 *(58.12)*
	250mg resveratrol with 20mg Piperine	1476.26 *(189.03)*	-243.72 *(67.01)*	-287.30 *(77.69)*	-375.74 *(94.44)*	-292.16 *(70.96)*
	Placebo	1475.04 *(161.35)*	-194.12 *(34.69)*	-149.39 *(70.65)*	-264.79 *(81.89)*	-271.44 *(57.14)*
RVIP correct (%)	250mg resveratrol	71.06 *(3.76)*	0.41 *(2.98)*	-4.48 *(2.44)*	-7.47 *(3.73)*	-7.76 *(2.58)*
	250mg resveratrol with 20mg Piperine	65.81 *(4.00)*	3.76 *(2.32)*	1.31 *(3.39)*	-4.36 *(3.39)*	-1.68 *(3.51)*
	Placebo	69.16 *(3.90)*	1.50 *(2.25)*	-7.38 *(3.65)*	-7.47 *(2.51)*	-6.66 *(3.40)*
RVIP Reaction Time (msec)	250mg resveratrol	494.24 *(8.87)*	5.10 *(5.73)*	0.61 *(8.76)*	1.18 *(7.86)*	3.90 *(9.57)*
	250mg resveratrol with 20mg Piperine	501.68 *(9.46)*	-7.17 *(8.07)*	2.06 *(10.99)*	-2.86 *(10.18)*	-4.06 *(9.98)*
	Placebo	499.22 *(0.13)*	-7.11 *(7.30)*	3.79 *(10.20)*	0.48 *(13.14)*	1.89 *(8.38)*

Table 2. Effects of 250mg resveratrol alone and when co-supplemented with 20mg piperine on mood in healthy, young human subjects. Mean values with their standard errors. N= 23. T= treatment; R= repetition. *=P<0·05, **=P<0·01.

Measure	Treatment condition	Baseline	Post-dose	ANOVA		
				Effect	F	P
Alert	250mg resveratrol	50.83 *(3.79)*	-6.65 *(5.44)*	T	.767	.470
	250mg resveratrol with 20mg Piperine	49.13 *(3.78)*	4.43 *(4.07)*	R	.359	.555
	Placebo	51.57 *(4.08)*	-4.87 *(4.68)*	T*R	3.28	.047*
Jittery	250mg resveratrol	16.83 *(2.91)*	19.78 *(5.40)*	T	.532	.591
	250mg resveratrol with 20mg Piperine	18.61 *(3.33)*	20.48 *(4.95)*	R	25.79	<.001**

	Placebo	15.39 *(2.54)*	20.87 *(4.73)*	T*R	.022	.979
	250mg resveratrol	28.96 *(4.69)*	35.65 *(6.18)*	T	.839	.439
Mental Fatigue	250mg resveratrol with 20mg Piperine	27.48 *(4.86)*	32.48 *(5.93)*	R	45.47	<.001**
	Placebo	26.22 *(4.10)*	33.74 *(6.11)*	T*R	.147	.864
	250mg resveratrol	62.87 *(3.46)*	-16.13 *(4.48)*	T	2.66	.081 t
Overall Mood	250mg resveratrol with 20mg Piperine	64.48 *(3.04)*	-12.78 *(3.60)*	R	25.87	<.001**
	Placebo	67.35 *(2.71)*	-13.74 *(2.97)*	T*R	.321	.727
	250mg resveratrol	62.91 *(2.67)*	-24.52 *(5.62)*	T	.566	.572
Relaxed	250mg resveratrol with 20mg Piperine	60.35 *(3.29)*	-14.13 *(6.00)*	R	20.70	<.001**
	Placebo	62.52 *(1.98)*	-20.61 *(4.44)*	T*R	1.79	.179
	250mg resveratrol	25.48 *(3.29)*	25.74 *(6.35)*	T	2.32	.110
Tense	250mg resveratrol with 20mg Piperine	23.87 *(3.28)*	26.35 *(6.40)*	R	26.08	<.001**
	Placebo	19.83 *(3.02)*	25.30 *(5.37)*	T*R	.016	.984
	250mg resveratrol	47.09 *(4.51)*	14.57 *(5.33)*	T	.405	.669
Tired	250mg resveratrol with 20mg Piperine	50.74 *(5.05)*	4.04 *(3.92)*	R	5.96	.023*
	Placebo	45.57 *(4.42)*	11.52 *(6.39)*	T*R	1.72	.191

Blood pressure

No significant, treatment related differences were observed on pulse rate, diastolic or systolic blood pressure. Raw baseline and change from baseline post-dose BP readings are displayed in table 3.

Table 3. Effects of 250mg resveratrol alone and when co-supplemented with 20mg piperine on blood pressure in healthy, young human subjects. Mean values with their standard errors in brackets.

Measure	Treatment condition	Task battery repetition			ANOVA		
		Baseline	PD 1	PD 2	Effect	F	P
Systolic Blood Pressure (mmHg)	250mg resveratrol	112 *(1.98)*	2.35 *(1.77)*	4.87 *(1.21)*	Tr	.621	.542
	250mg resveratrol with 20mg Piperine	114.17 *(1.98)*	1.39 *(1.26)*	4.90 *(1.72)*	Ti	9.61	.005**
	Placebo	113.22 *(2.31)*	-0.04 *(1.78)*	3.39 *(2.13)*	Tr*Ti	.089	.915

Diastolic Blood Pressure (mmHg)	250mg resveratrol	75.65 *(1.66)*	2.57 *(0.90)*	4.17 *(0.96)*	Tr	3.68	.045*
	250mg resveratrol with 20mg Piperine	75.09 *(1.62)*	4.83 *(1.38)*	4.70 *(1.65)*	Ti	.628	.437
	Placebo	76.91 *(2.48)*	-0.17 *(2.08)*	0.65 *(1.77)*	Tr*Ti	.258	.724
Pulse Rate (BPM)	250mg resveratrol	68.43 *(2.48)*	-0.83 *(1.07)*	-2.26 *(1.51)*	Tr	1.77	.192
	250mg resveratrol with 20mg Piperine	67.91 *(2.14)*	0.35 *(1.87)*	-3.74 *(3.78)*	Ti	3.38	.080 t
	Placebo	70.87 *(2.29)*	-3.78 *(1.63)*	-6.87 *(1.63)*	Tr*Ti	.368	.584

PD, post-dose; Tr, treatment; Ti, time; bpm, beats per min.; t, trend. There were significant main effects for Tr and Ti: *P,0·05, **P,0·01. † Baseline, immediately before treatment; PD 1, 40min post-dose and immediately before post-dose tasks; PD 2, 95 min post-dose and immediately after post-dose tasks.

Bioavailability

No resveratrol (in any form) was found in baseline samples, indicating that all volunteers did not consume resveratrol before the study. Following oral intervention with 250mg of resveratrol, plasma concentrations of total resveratrol metabolites ranged from 2-18.2μM, varying between individuals and treatments. However, no aglycone *trans*- or *cis*-resveratrol was quantifiable in plasma. Resveratrol 3-*O*-sulfate was the predominant metabolite in all volunteers, contributing 59-81% of total metabolites. The 4'- and 3-*O*-glucuronide forms made roughly equal contributions to the remaining metabolites in circulation. C_{max} was typically achieved at 90 minutes. Resveratrol conjugates were present in plasma as both *trans*- and *cis*-isomers, varying between individuals. The average C_{max} *trans*- :*cis*-ratios for resveratrol 3-*O*-sulfate and resveratrol 3-*O*-glucuronide following consumption of all *trans*-resveratrol were 4.7±5.6 (ranging 1.2-15.9) and 5.1±5.6 (ranging 0.94-18.8), respectively. *Cis*-resveratrol 4'-*O*-glucuronide was observable within some, but not all subjects. Extraction efficiency tests did not indicate significant induction of isomerization during sample handling, suggesting that this conversion occurs *in vivo*.

Whilst average concentrations at C_{max} for resveratrol 3-*O*-sulfate, 4'-*O*-glucuronide and 3-*O*-glucuronide appeared lower following piperine co-supplementation compared to resveratrol alone, there was no significant difference between treatments. Similarly, there was no significant difference for area under the curve values, and T_{max} was not significantly changed between treatments.

Mean plasma concentrations of *trans*-resveratrol 3-*O*-sulfate and combined 4'-*O*-glucuronide and 3-*O*-glucuronide metabolites at pre-treatment and at 45-, 90- and 120 minute post-dose time-points, for both treatment conditions, are shown in Figure 3.

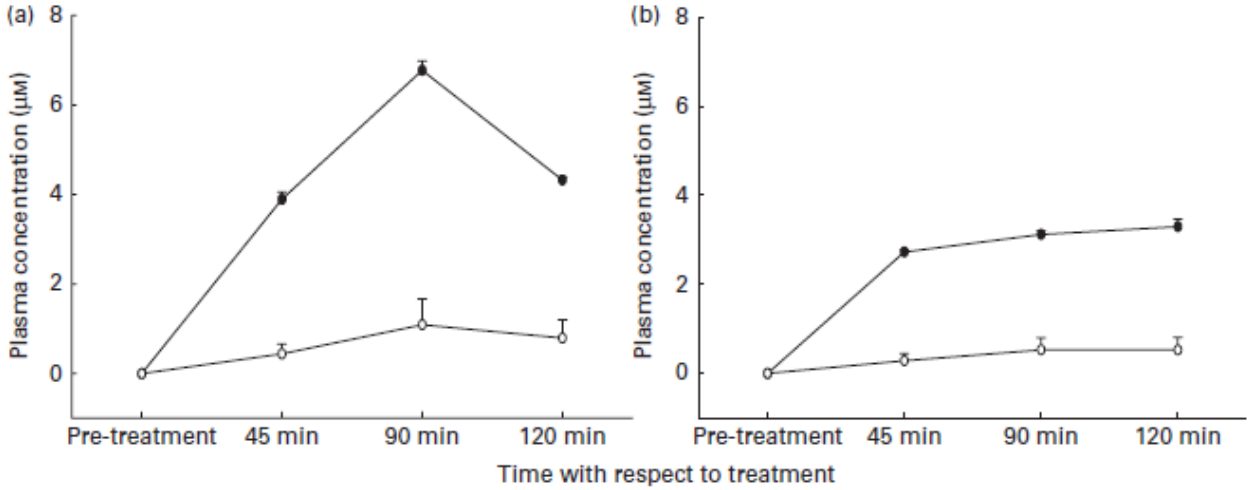

Fig. 3. Plasma bioavailability of resveratrol metabolites following (a) the administration of 250mg trans-resveratrol alone and (b) the administration of 250mg trans-resveratrol with 20mg piperine in healthy, young human subjects. Values are means (n 6), with their standard errors represented by vertical bars. ●, Concentration of resveratrol 3-O-sulphate; ○, combined concentrations of resveratrol 40-O-glucuronide and resveratrol 3-O-glucuronide.

DISCUSSION

The current study demonstrates that the well-established bioenhancer piperine, is also able to increase the bioefficacy of the polyphenol resveratrol when co-supplemented in healthy humans. Whereas 250mg orally administered *trans*-resveratrol had no significant effects on overall CBF (total-Hb) during cognitive task demands, co-administration of the same dose of resveratrol with 20mg piperine resulted in significantly increased CBF for the duration of the 40 minute post-dose task period. The findings with regards resveratrol alone in this respect are broadly in line with the dose response pattern of CBF evinced following resveratrol in a previous study; in which 250mg was largely ineffective [10]. Despite this piperine-mediated enhancement of resveratrol's CBF effects however, there were no significant treatment related differences in performance of the cognitive tasks nor on blood pressure/heart rate, or participants' ratings of mood for either active treatment.

The pattern of hemodynamic effects of resveratrol seen here, when enhanced with piperine, is exactly in line with the aforementioned previous resveratrol intervention study following a 500mg dose [10]. This pattern is seen as significantly higher levels of total- and oxy-Hb, alongside deoxy-Hb, during the post-dose cognitive task period and represents increased CBF and oxygen utilization respectively. This hemodynamic response is dissimilar to that seen during cognitive task

393 performance alone. Here total- and oxy-Hb typically rise alongside a concomitant decline in deoxy-
394 Hb levels [28], with this phenomenon predicated on the fact that neural activation instigates an
395 increase in CBF which is greater than the metabolic rate of oxygen extraction/utilization. As such,
396 deoxy-Hb can be observed to decrease during cognitive performance [29]. The different deoxy-Hb
397 response seen following resveratrol is likely predicated on indirect effects on mitochondrial
398 phosphorylation. In support of this, Lagouge et al. [30] report that, in mice, supplementation with
399 400mg/kg/day resveratrol, for 15-weeks, significantly increased mitochondrial structures and
400 enzymatic activity. This resulted in a significant increase in O_2 consumption and VO_2 max rate and
401 was observed to increase running time and tolerance to cold. In terms of mechanisms, resveratrol is
402 able to interact with the sirtuin ('silent information regulator': SIRT) system; a class of proteins
403 involved with multifarious biological processes that has received a great amount of attention over
404 the past decade in relation to life-extension [31]. Of importance here, SIRT is implicated in the
405 deacetylation of Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α); a
406 gene which controls mitochondrial biogenesis and function [32], and, whilst the oxygenation effects
407 in the above study in rodents followed chronic consumption, these mechanisms would explain the
408 O_2 consumption effects seen here; represented by deoxy-Hb.

409 Interestingly, in light of significant CBF effects occurring only with the resveratrol/piperine
410 combination, no significant differences were found in plasma levels of resveratrol between
411 treatments. In both treatment conditions, resveratrol metabolites were present in plasma across the
412 post-dose cognitive task period and the parent compound was un-quantifiable at all time points.
413 However, contrary to the hypothesis of piperine-induced bioenhancement, the pattern of effects here
414 actually suggests inhibition rather than enhancement of plasma levels, e.g. the C_{max} of total
415 metabolites after 250mg resveratrol was 9.98μM compared to 4.82μM in the piperine co-
416 supplemented condition. Piperine also appeared to be inhibiting the transit of resveratrol; evidenced
417 by the t_{max} of metabolites in the 250mg resveratrol condition occurring at the 90 minute sample
418 time-point compared to the 120 minute time-point in the co-supplemented condition and the
419 observation of metabolite levels reducing at the 120 minute time-point in the 250mg resveratrol
420 condition and not in the co-supplemented group. Nevertheless, this pattern of effects evinced no
421 significant differences between treatment groups which suggests two possibilities; either piperine is
422 able to exert CBF effects independently of resveratrol or, alternatively, it potentiates the effects of
423 resveratrol seen previously on CBF.

424 Taking the first of those possibilities then, it is notable that only 1 study [33] exists to suggest that
425 piperine is capable of interacting with NO and that this was the inducible NO synthase isoform

(iNOS) which is stimulated in response to immunological stimuli [34] and is not associated with cerebral vasorelaxation and increased blood flow. No data exist to suggest that piperine is capable of affecting oxygenation, or indeed any other factor relevant to this study, and, taken together, this precluded the need for a piperine-only treatment condition here. The exception here is a small amount of literature in rats which suggests that chronic (up to 4-weeks) piperine supplementation might improve aspects of performance; although this appears to be mostly related to mood augmentation rather than enhanced cognition per say [35-37]. Nevertheless, future studies may warrant investigation of the efficacy of piperine alone on these parameters, in humans, in order to clarify this issue.

In light of a lack of evidence to suggest that piperine has any influence on parameters relevant to CBF, and in the face of no significant modulation of CBF in the resveratrol condition alone (a finding mirrored in Kennedy et al. [10] with the same dose) it seems more likely that piperine is increasing the bioefficacy of resveratrol by potentiating its vasorelaxatory properties. In support of this, resveratrol is a well validated vasorelaxatory mediator [7] and, at a higher dose (500mg), can increase CBF in healthy humans [10].

Of the potential mechanisms to explain the efficacy enhancing effects of piperine, one possibility is that piperine is able to enhance the activity of resveratrol, the neuronal vasculature, and/or some other factor relevant to CBF via thermogenic properties. As evidence of piperines' heat-proffering properties, specifically in neural tissue, Reanmongkol et al. [38] report on the ability of piperine to stimulate activity of ATPase (but inhibition of oxidative phoshorylation) which produces heat as a by-product [39]. Thermogenic increases in tissue activity have previously been proposed as an explanation for piperine-mediated increases in plasma beta-carotene levels in humans [13] via increasing the absorption rate of the intestinal epithelium and, as a mechanism, could exist without piperine evincing an overall increase in resveratrol bioavailability: a phenomenon observed previously [11-14] but not replicated here.

In terms of behavioural effects, the results of the current study are in line with previous findings: i.e. a lack of any effect of a 250mg dose of resveratrol with regards cognitive task performance [10]. One of the primary reasons for utilizing piperine here was to investigate whether this well established bioenhancer of polyphenols might also evince an enhancement of resveratrol's bioefficacy; especially in terms of cognitive function due to the null effects reported previously. However, whilst the increase in CBF during task performance was potentiated by piperine, the pattern was largely the same as that seen following a larger dose of resveratrol (500mg in [10]) where cognitive

effects were also lacking. It would therefore appear that acute increases in CBF are not sufficient, in themselves, to alter cognitive function in the young, healthy cohorts utilized here and previously. However, it may be the case that longer-term supplementation is required, or indeed that the effects might translate into cognitive benefits in populations showing age- or pathology-related decrements in CBF and cognitive function.

In conclusion, this is the first study to report that piperine co-supplementation enhances the bioefficacy of resveratrol with regards CBF effects in healthy humans, but not cognitive performance, and does this without altering the overall bioavailability of resveratrol *in vivo*.

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No financial support was received for this study. The treatments and other materials were purchased on the open market.

CONFLICTS OF INTEREST

None of the authors has any conflict of interests with regards the research described in this paper.

AUTHORSHIP

All of the authors (DK, EW, CH, JR, GW & TD) were actively involved in the planning of the research described herein and in writing the paper. EW collected the data. GW and TD planned and carried out the analysis of the plasma samples. All authors contributed to and reviewed the final publication.

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