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Review

Iron and Physical Activity: Bioavailability Enhancers, Properties of Black Pepper (Bioperine®) and Potential Applications

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Abstract: Black pepper (*Piper nigrum* L.) has been employed in medicine (epilepsy, headaches, and diabetes), where its effects are mainly attributed to a nitrogen alkaloid called piperidine (1-(1-[1,3-benzodioxol-5-yl]-1-oxo-2,4 pentenyl) piperidine). Piperine co-administered with vitamins and minerals has improved its absorption. Therefore, this study aimed to describe the impact of the joint administration of iron (Fe) plus black pepper in physically active healthy individuals. Fe is a micronutrient that aids athletic performance by influencing the physiological functions involved in endurance sports by improving the transport, storage, and utilization of oxygen. Consequently, athletes have risk factors for Fe depletion, Fe deficiency, and eventually, anemia, mainly from mechanical hemolysis, gastrointestinal disturbances, and loss of Fe through excessive sweating. Declines in Fe stores have been reported to negatively alter physical capacities such as aerobic capacity, strength, and skeletal muscle recovery in elite athletes. Thus, there is a need to maintain Fe storage, even if Fe intake meets the recommended daily allowance (RDA), and Fe supplementation may be justified in physically active individuals, in states of Fe deficiency, with or without anemia. Females, in particular, should monitor their Fe hematological profile. The recommended oral Fe supplements are ferrous or ferric salts, sulfate, fumarate, and gluconate. These preparations constitute the first line of treatment; however, the high doses administered have gastrointestinal side effects that reduce tolerance and adherence to treatment. Thus, a strategy to counteract these adverse effects is to improve the bioavailability of Fe. Therefore, piperine may benefit the absorption of Fe through its bioavailability enhancement properties. Three research studies of Fe associated with black pepper have reported improvements in parameters related to the metabolism of Fe, without adverse effects. Although more research is needed, this could represent an advance in oral Fe supplementation for physically active individuals.

Keywords: iron; bioavailability; black pepper; piperine; supplementation; physical activity

1. Introduction

Iron (Fe) is the most abundant trace mineral involved in cell metabolism and the growth of organisms. A smaller fraction (2%) localized in some proteins containing heme and Fe is present as Fe-sulfur (S) groups that contribute to physiological systems such as oxygen (O_2) transport,

DNA synthesis, metabolic energy, cellular respiration and electron transport in mitochondria. Approximately 30% and 10% of body Fe is stored as ferritin (Ft) and hemosiderin in the liver, bone marrow, and muscle. In addition, Fe can be used in erythropoiesis according to the demands of the body [1].

Physical activity (PHA) causes alterations in different cellular metabolic processes, which are reflected in modifications in biochemical and/or hematological parameters. Thus, the concentration and availability of Fe in the body is an essential factor that conditions aerobic capacity in PHA healthy individuals. Fe is a crucial mineral involved in the different physiological mechanisms related to physical performance and resistance, particularly in endurance sports. Therefore, Fe is involved in the process of oxidative phosphorylation and in the activity of antioxidant enzymes involved in muscle strength and endurance. In addition, Fe is a component of hemoglobin (Hb), which is a marker of blood O_2 capacity linked to the sports results. Myoglobin (Mb), with a Fe atom in its structure, ensures O_2 supply during intensive physical exercise, and exhibits antioxidant properties [2].

Thus, Fe depletion, with or without anemia, may impair aerobic physical performance. A decrease in maximal oxygen uptake during PHA has been found as a consequence of iron deficiency (ID). Indeed, marginal ID provokes a reduction in endurance capacity, only when an impoverishment of tissue and a drop in serum Ft concentration have occurred. Furthermore, Fe homeostasis is altered in some athletes competing in and training for endurance sports. In this sense, it has shown that decreased Fe storage increases fatigue, delays skeletal muscle recovery, and decreases strength and aerobic capacity. In addition, serum Fe and transferrin saturation are changeable depending on the degree of fatigue and exercise training load [3,4].

Clinical signs of ID are the most frequent cause of triggering *true anemia* in athletes [3]. However, *sports anemia*, which was first reported by Yoshimura et al. [5] is due to lower Hb concentrations than the sedentary population. This *sports anemia* is the result of a physiological response to aerobic physical exercise caused by a volume of expanded plasma that dilutes erythrocytes. *True anemia* in athletes limits sports performance, derived from prolonged strenuous exercise that directly affects Fe metabolism and reduced Hb and Ft [3]. In addition, several etiological factors may explain storage Fe depletion in PHA healthy individuals such as gastrointestinal blood loss, increased loss of Fe in sweat and urine, intestinal Fe malabsorption and malnutrition, mechanical hemolysis, and menstruation is an added risk factor for ID [6]. Furthermore, a mechanism of physical activity-induced ID involving the influence of inflammation associated with the development of PHA on the post-exercise hepcidin (HAMP) response has been proposed. The impact of PHA on HAMP-dependent control of Fe status by cytokine-induced HAMP up-regulation represents a mechanism behind ID (possibly leading to anemia) in PHA active, healthy individuals and may have important practical implications for physical performance [7].

For this reason, it is essential to maintain reserves of Fe to maintain sports performance. Therefore, supplementation may be justified in PHA active, healthy individuals, even when the diet meets the recommended daily amount for Fe [4,8,9]. Furthermore, supplementation with Fe is the most commonly used strategy to achieve adequate levels of Ft and/or avoid ID in PHA active, healthy individuals [10].

These facts may make sports nutrition researchers consider new strategies for Fe supplementation. Among these ergo-nutritional strategies is the simultaneous administration of foods whose active ingredients stimulate absorption, increasing bioavailability, and reducing the dose and, therefore, the adverse effects of Fe supplementation [11]. In this context, black pepper may be useful because piperine (alkaloid present in black pepper) co-administered with vitamins and minerals has been shown to improve their absorption. Therefore, this manuscript aims to describe the impact of co-administration of Fe plus black pepper, as a commercial preparation Bioperine®, on physically active healthy individuals.

2. Dietary Iron

Adequate nutritional intake of Fe has been established as the first control measure in ID. The absorption of heme Fe should be considered higher than that of free Fe. The two states of free Fe also have differences in intake, with ferrous ion (Fe^{2+}) more widely absorbed than ferric ion (Fe^{3+}) [12]. In the diet, the bioavailability of Fe varies between 5–15%, depending on the body's reserves of Fe. In ID, the bioavailability of Fe increases to 35%. In addition, Table 1 shows that the absorption of Fe in the intestinal tract conditioned by different nutritional enhancers and inhibitors [13].

Table 1. Nutritional Enhancers and Inhibitors of Iron Absorption.

Nutritional Enhancers	Nutritional Inhibitors
Vitamin C	Phytates
Peptides from partially digested muscle tissue	Oxalates
Fermented food	Polyphenols: coffee and black tea
Organic acids: malate or citrate	Peptides from partially digested vegetable proteins
Meat	
Fish and Shellfish protein	Minerals: calcium

Although the recommended dietary allowance (RDA) of Fe is 15 mg/day for women and 10 mg/day for men, the estimated average requirement (EAR) should be increased by 30–70% because of the etiological factors that we have reported on in another section of this manuscript concerning athletes [10]. A diet of approximately 2500 kcal is appropriate for athletes who provide 2.3 mg Fe/day. In addition, these diets include the energy intake needed for athletes who most often suffer from ID, which may be related to their low body mass index [14,15].

3. Benefits of Oral Iron Supplementation

The pharmaceutical oral forms of Fe preparations are non-heme (iron salts) and heme (higher bioavailability). Oral supplements with ferrous salts (sulfate, fumarate, and gluconate) are better absorbed (10–15% bioavailability) than ferric salts of Fe complexes that are administered together with amino acids, polysaccharides, and proteins such as ovalbumin. However, high adverse gastrointestinal effects such as constipation, nausea, vomiting, diarrhea, and dark stools have been reported for ferrous Fe salts [16,17].

The amount of elemental Fe that is available to be absorbed into the body varies depending on the iron salt. Thus, the amount of elemental Fe is 20% for ferrous sulfate ($FeSO_4$), 33% for ferrous fumarate, and 12% for ferrous gluconate. We must consider that if 100 mg of $FeSO_4$ is equivalent to 20 mg of elemental Fe, this is approximately the RDA (18 mg/day).

It has described that 50% of patients supplemented with oral Fe have gastrointestinal adverse effects induced by the direct toxicity of ionic Fe due to its caustic action on the intestinal mucosa [18]. It should also have considered that the excess of Fe, due to the abuse of supplements, makes it difficult for the organism to get rid of Fe micronutrients caused by Ft saturation. The excess of Fe is stored in the form of hemosiderin, which causes hemosiderosis (deposits of Fe in the liver or spleen) and hemochromatosis (deposits of Fe in body tissues) [1]. As a consequence of these adverse reactions, there is a reduction in tolerance and adherence to the traditional treatment with oral Fe salts [19].

Furthermore, heme Fe supplements have a lower incidence than non-heme Fe, which could be through different routes in the absorption mechanism. Non-heme Fe by the action of stomach hydrochloric acid passes into its reduced form, Fe^{2+}, which is the soluble chemical form capable of passing through the membrane of the intestinal mucosa. Although Fe can be absorbed throughout the entire intestine, its absorption is most efficient in the duodenum and the upper part of the jejunum. The intestinal mucous membrane has the facility to trap the Fe and allow its passage inside the cell, due to the existence of a specific receptor in the layer of the edge in the brush. The apotransferrin of the cytosol contributes to increasing the speed and efficiency of Fe absorption. This type of heme Fe crosses

the cell membrane as an intact metalloporphyrin, once the endo-luminal or enterocyte membrane proteases hydrolyze the globin. The products of this degradation are essential for the maintenance of heme in a soluble state, thereby ensuring its availability for absorption [10].

Generally, 100–200 mg taken once or twice a day is prescribed, which is a high dose of elemental Fe [20]. The adverse reactions can be minor when a lower, more frequent, dose is dispensed. In this sense, one possibility is to use 100 mg of $FeSO_4$ taken as 50 mg twice per day [19]. A daily dose of between 50 mg/day and 150 mg/day 1 may be adequate for non-anemic athletes. However, Villanueva et al. [19] used very high doses of up to 300 mg/day in bleeding patients with severe ID anemia. We have reported [4] that oral supplementation with 325 mg/day of ferrous sulfate as 105 mg/day of elemental Fe for 11 weeks does not modify Fe reserves and increases the strength of elite volleyball players. In addition, Cordova et al. [16] prevented the decrease of serum Fe, Ft, Hb and hematocrit, and improved recovery in elite cyclists during the *Vuelta a España* competition by administering 800 mg Fe protein succinate, which contained 80 mg/day Fe. In this sense, Nielsen et al. [21] showed that a fasting dose of 100 mg/day Fe ferrous administered for 3 months or more is suitable for non-anemic athletes with low serum Ft or ID only and also in anemic athletes.

An effective treatment for ID is supplementation with a dose of 60–80 mg/day of elemental Fe for 12 weeks, in healthy persons [22]. In this sense, the use of 80 mg/day of Fe achieved a decrease in fatigue in non-anemic menstruating women with low Ft [23]. However, another author [24] recommended an intake of heme Fe including meat, fish, legumes, and vegetables five times a week for healthy non-anaemic people with ID. Mielgo et al. [25] reported that an intake of 25.8 mg/day of dietary Fe is not sufficient to prevent 30% of female volleyball players from suffering from pre-latent Fe deficit and 20% from latent (pre-anemia) deficit. However, this does not guarantee high levels of Fe. Therefore, these dietary recommendations were supplemented with 28 to 50 mg of elemental Fe daily with mild gastrointestinal adverse effects [24].

4. Bioavailability of Dietary Iron

One strategy to counteract these adverse effects derived from supplementation and combat Fe ID is to improve the bioavailability of Fe. Bioavailability, defined as the efficiency with which Fe is obtained from the diet, is biologically used, and considered when it is necessary to enhance dietary intake [26].

Some factors modulate the bioavailability of dietary Fe, such as the content of Fe-hemic and Fe-non-hemic in foods; the original state of Fe, considering that, in an oxidized state and at a pH > 4.0, it is exceedingly insoluble and scarcely absorbed; the metabolic demand of Fe; the substances present in the diet that can be promoters that stimulate the absorption (nutritional enhancers) or that block the absorption of Fe (dietary inhibitors); the interactions between Fe and the components of the digestive tract [27]; the genetic endowment of the individual associated with the absorption of Fe, which determines the levels of Fe in serum and is determined by genes such as HFE, TMPRSS6, TFR2 and TF [28].

The bioavailability of Fe is also conditioned by some physiological factors such as body reserves of Fe, the speed of erythropoiesis, hypoxia, infections, and the mobilization of Fe and HAMP, which is a hepatic peptide intimately related to the homeostasis of this micronutrient [1,13]. Thus, there are interactions between exercise-induced factors and the expression of HAMP in humans, which are directly related to an increase or decrease in the absorption and bioavailability of Fe. Factors that negatively regulate exercise-related HAMP levels include anemia, hypoxia that triggers erythropoietin (EPO) secretion, and hemolysis [29]. On the other hand, inflammation, oxidative stress and interleukin-6 (IL-6) act by positively regulating the levels of HAMP [30]. These factors are induced during exercise, causing an alarming increase in HAMP, mainly as an acute-phase inflammatory response. Therefore, the origin of a negative iron balance in athletes may be due to factors related to exercise (type, duration, and intensity) and HAMP status. All of these directly affect iron absorption and hematological parameters. HAMP expression decreases metal absorption, increases cytoplasmic concentrations

of Fe-saturated Ft, and decreases transport to the blood vessels, whereby Fe accumulates in the enterocyte and is excreted within the desquamated epithelial cells from the intestine. On the other hand, unlike excesses of Fe, deficiencies of Fe, hypoxia, and states of increased erythropoiesis generate a decrease in the expression of HAMP, and therefore, these situations are directly related to an increase in the absorption and bioavailability of Fe [13,31].

Some studies [32–35] indicate that the concomitant use of vitamin C (ascorbic acid), vitamin A, vitamin E, vitamin B9 (folic acid), vitamin B12 (cobalamin) and vitamin D3 (cholecalciferol) increases the bioavailability and absorption of Fe in the intestinal tract. These absorption enhancers can be provided by diet or direct supplementation preparations. These compounds could lead to formulations of multi-components associated with Fe that would facilitate its absorption in the digestive tract [36].

In this way, vitamin C is a promoter of the absorption of non-heme Fe. However, it does not affect heme Fe. Its effects are related to its reducing power, which blocks the synthesis of insoluble ferric hydroxide, and also its the capacity to synthesize soluble complexes with ferric ions, which maintains solubility at an alkaline pH in the duodenum. Similarly, other promoters, such as vitamins A and E, act in a similar pathway [37].

Mielgo-Ayuso et al. [35] described a positive relationship between 25(OH)D levels and levels of faith in the body in a group of elite athletes. This study reports that vitamin D3 stimulates erythropoiesis, probably because reticulocytes express receptors for the active form of vitamin D3 that would accelerate their proliferation and maturation into erythrocytes. Thus, supplementation with vitamin D3 would play a vital role in the prevention of ID and/or anemia.

The *naive* erythrocytes formed recently by erythropoiesis allow *senior* erythrocytes to be replaced daily by phagocytosis. Koury et at. [38] described the critical role that Fe, folate, and vitamin B12 play in erythropoiesis. Folate and vitamin B12 stimulate erythrocyte ontogeny from the erythroblast stage. Thus, folate and vitamin B12 deficiency inhibit purine and thymidylate synthesis, impair DNA synthesis and cause erythroblast apoptosis, resulting in ineffective erythropoiesis. The consequences of which produce anemia.

It is necessary to consider in the supplementation that the pharmaceutical form of Fe administered has a decisive influence on bioavailability. Biopharmaceutical bioavailability includes the availability, absorption, retention, and use of Fe, and is the critical factor for Fe to be biologically productive [39]. Fe preparations with higher bioavailability, which allow one to take lower absolute doses of Fe, may cause less gastrointestinal discomfort and be better tolerated by patients [21,39]. One way to increase the bioavailability of Fe is through the use of absorption enhancers, which have significant repercussions in terms of the pharmacokinetics and pharmacodynamics of Fe supplements, improving the processes of release, absorption, metabolism, utilization, and excretion [25].

5. Characteristics of Black Pepper as a Bioavailability Enhancer

Black pepper (*Piper nigrum* L.), is used in traditional medicine because it contains the active pharmaceutical ingredient 1-(1-[1,3-benzodioxol-5-yl]-1-oxo-2,4 pentenyl) piperidine, which is a pungent nitrogen alkaloid. Black pepper is used to treat epilepsy, headaches, and diabetes. In addition, *Piper nigrum* L. is used in the cooking of some foods with a unique flavor and as a preservative in food additives or perfumes [40].

Piperine has a low risk of toxicity, is not genotoxic and does not present any significant adverse effects in the murine model at supraphysiological doses (5–20 times higher than the average human intake) on internal organs, weight, Hb levels, total serum proteins, albumin, cholesterol, fats, and initrogen. Its various medical properties and advantageous safety profiles facilitate the pharmaceutical employment of piperine as a dietary and health supplement. As a result, BioPerine® capsules (Sabinsa Corporation, East Windsor, NJ, USA), which are a black pepper extract commercially available as a dietary supplement, are formulated up to 15 ppm as a food ingredient [40,41].

Bioperine® has been administered together with vitamins, minerals, and some nutrients (Table 2; extracted from Majeed M et al. [42]) as a bioavailability enhancer [42]. The heat effect is a manifestation

of the biological activity of the piperine contained in the black pepper. Bioperine®, a standardized extract of *Piper nigrum* L. containing 95% piperine in the form of 1-piperopiperidine, stimulates thermogenic action in the epithelial cells of the small intestine. This acts as a thermonutrient that allows for increased absorption and bioavailability of the nutrients [42–44].

Table 2. Nutritional elements co-administrated with BioPerine® that increase absorption.

Group Elements	Nutritional Elements
Herbal Extracts Curcuminoids	Curcuminoids, Boswellia Serrata Extract, Ashwagandha, Ginkgo Biloba Extract, Capsaicin, Bioflavonoids.
Water-soluble Vitamins	B1, B2, B3, B6, B9, B12, C.
Liposoluble Vitamins	A, D, E, K.
Antioxidants	vitamin A, vitamin C, vitamin E, alPHA-carotene, beta-carotene, beta-cryptoxantine, Lycopene, lutein/zeaxanthin, pine bark bioflavonoid complex, Ge, Se, Zn.
Amino acids	lysine, isoleucine, leucine, threonine, valine, tryptoPHAn, phenylalanine and methionine
Minerals	Fe, Zn, V, Se, Cr, I, K, Mn, Cu, Ca, Mg.

The mechanism of action (Figure 1) responsible for the bioavailability enhancing effect of Bioperine® is because a small amount of piperine stimulates the release of catecholamines, thermogenic hormones whose activity is made possible by the presence of cyclic adenosine monophosphate (cAMP) [42,43]. The thermogenic effect has been localized in the epithelial cells of the intestine that increase the absorption of nutrients. However, the catecholamine-mediated thermogenic response is relatively short. Therefore, the window of absorption has a narrow temporal scope. These thermogenic properties may explain how a minimal amount of BioPerine® can produce such a powerful effect on the uptake of serum nutrients [45]. The possible justification for this mechanism would be due to modifications in the concentration of cholesterol (the central element of the plasma membrane) in the cell membranes, altering the fluidity of the lipid bilayer, and some functions of the layer such as some enzymatic activities and the processes of ion transport. In a previous study, piperine significantly lowered cholesterol concentrations in the villi. However, it did not affect the phospholipid content of the intestinal villi, resulting in a relatively lower cholesterol: phospholipid ratio in the villi of the jejunum and ileum. Those changes in the villi lipid profile might be linked to increasing the lipid fluidity of the membranes of the jejunum and ileum. These alterations of membrane fluidity directly influence the passive permeability properties of layers [46].

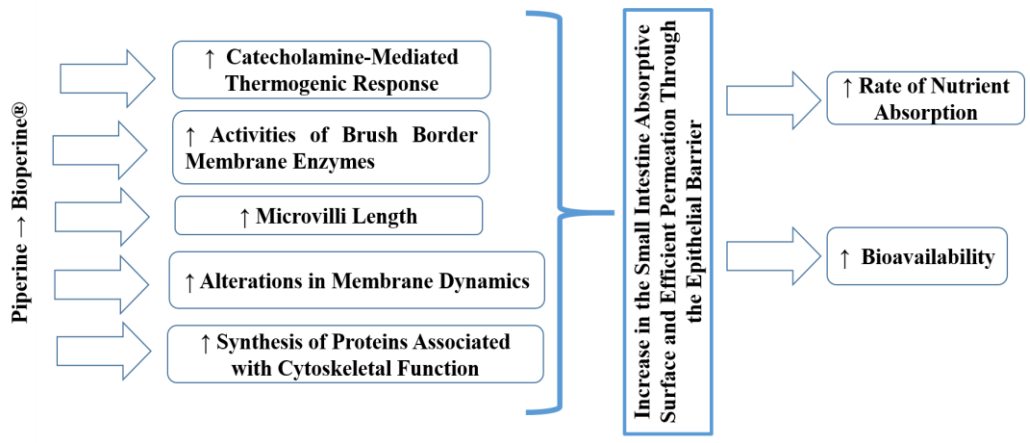

Figure 1. Action mechanism of piperine/Bioperine® as a bioavailability enhancer.

In another study, black pepper and piperine aroused the activities of brush border membrane enzymes, including glycyl-glycine dipeptidase, leucine aminopeptidase, g-glutamyl transpeptidase and alkaline phosphatase in the jejunal mucosa, which stimulate the active transport of amino acids. There are other pathways by which piperine stimulates nutrient absorption, including increased micelle formation and modification of the epithelial cell membrane due to piperine's affinity to fatty substances [43]. This alteration in the dynamics of membrane lipids, together with the change in the conformation of enzymes in the intestine, would also facilitate the absorption of Fe [44]. Taken together, these results may suggest that piperine alters membrane structure by taking advantage of the apolar nature, allowing it to interact with surrounding lipids and hydrophobic portions of proteins. Perhaps all these interactions will overcome the steric hindrance of membrane lipids to proteins and thus modulate enzyme formation.

Furthermore, ultrastructural analyses with electronic scanning microscopy revealed an increase in microvilli length in the presence of piperine. It was suggested that piperine improves the permeability of the epithelial barrier of intestinal cells by altering their plasma membrane. In addition, piperine can induce the synthesis of cytoskeleton-related proteins that cause an increase in the absorption surface of the small intestine [43,44].

6. Effectiveness of BioPerine® as a Thermonutrient (Stimulating the Bioenergetic Processes)

BioPerine® with thermonutrient activity and bioavailability enhancement properties is a natural product. A thermonutrient is a nutrient with properties stimulated the bioenergetic processes in the gastrointestinal epithelium [47]. It has been observed that the administration of a preparation of beta-carotene (15 mg) plus Bioperine® (5mg) increases the levels of beta-carotene by approximately 2 times in humans. Coenzyme Q10, L-(+)-selenomethionine, vitamin B6, vitamin C, and plant-based compounds, such as curcumin extract, showed improved bioavailability when given in partnership with BioPerine®. The administration of Bioperine (5 mg) to the preparation of vitamin C plus isopropanol only increased the bioavailability of vitamin C, but the bioavailability of alcohol was not altered [43].

With respect to Fe, Majeed et al. [45] evaluated the bioavailability of elemental Fe in combination with and without BioPerine® in an animal model, with the objective of validating the capacity of BioPerine® in improving the bioavailability of oral Fe. The animals were stable and followed the same diet, based on feed, provided by the animal. They found that the Fe serum concentration was significantly higher in rabbits in the Fe plus BioPerine® group (36.55 ± 9.97 μg/mL at 8 h) compared to the control group, Fe without BioPerine® (4.730 ± 0.94 μg/mL at 24 h); thus confirming that BioPerine® could be employed as a critical natural product to increase the bioavailability of elemental Fe.

Majeed et al. [44] performed a study on regular physical activity practitioners with ID and anemia. Fe supplementation with BioPerine® showed significant efficacy for 56 days, evaluated by increased Hb levels ($p \leq 0.0001$), and it also decreased the erythrocyte sedimentation rate ($p \leq 0.001$). An increase in serum Fe may be correlated with the immediate release of Fe from the BioPerine® study supplement into the systemic circulation, thereby increasing the bioavailability of Fe over a substantial space of time. In addition, these authors described the increased health status of subjects in response to the SF-36 Health Questionnaire ($p \leq 0.0001$) and the decreased fatigue ($p \leq 0.0001$) assessed by the Fatigue Severity Scale during the study. A significant increase ($p < 0.05$) was observed in the Fe group with BioPerine® in the levels of Hb, Ft, and total Fe fixation capacity concerning the group-administered Fe. There were no significant differences ($p > 0.05$) in the diet between both groups, evaluated by a nutritionist using the Food Frequency Questionnaire (FFQ). Therefore, the administration of Fe and BioPerine® is potentially useful for the management of ID with anemia [44].

Recently in professional rowing athletes, Fernández-Lázaro et al. [36] evaluated the comparative efficacy of two oral Fe supplements, one supplement with a dose of 325 mg in the form of ferrous sulfate and another with a dose of 50 mg in the presence of BioPerine® during the 10-week sports season. The athletes had more than 3 years of experience in the elite rowing team of the Rowing

Club Association and took part in this randomized, non-placebo-controlled trial. The professional rowers followed a balanced diet supervised continuously by the club dietitian/nutritionist and all completed an analogous training program managed by the same graduate in Physical Education. Both supplements showed significant improvement in Ft compared to the control group, but not in sports performance. Thus, the possibility of obtaining Ft improvements through the use of six-times lower doses, together with bioavailability enhancers, could improve the risk/benefit ratio in the supplementation of athletes.

It should be noted that in the two human studies [36,44], none of the recruited subjects reported adverse events resulting from the use of Fe with BioPerine®, which indicates the safety and effectiveness of the supplement. However, in the future, more research is needed to re-evaluate the effectiveness of Fe and BioPerine® co-administration on the hematological profile and to determine possible improvements in sports performance over more extended periods of supplementation in different sports modalities.

7. Safety Verification for Black Pepper Intake

Standard daily intake levels, and even 250-times higher levels, of either black pepper or its active ingredient piperine showed no adverse effects on the parameters evaluated, such as growth, organ weight, and blood components [48]. The same was found in the murine model when rats were given doses of 5 to 20 times the healthy human intake. Other parameters were also evaluated, such as total serum protein, albumin, globulin, glucose, and cholesterol, activities of serum aminotransferases and phosphatases, fat and nitrogen balance, all of which were unaltered [49]. Concerning the possible genotoxicity of piperine, all tests performed showed the non-genotoxic nature of piperine [50]. Furthermore, none of the doses of piperine administered for five consecutive days to test the immunotoxic effect demonstrated any toxicity on the immune system [51]. For these reasons, we believe the black pepper or piperine, had no apparent toxic effect and does not appear to be a poisonous chemical.

The black pepper or its active ingredient, piperine, has been reported in this manuscript to have the potential to increase the bioavailability of some nutrients, especially iron ore, andhas low toxicity. However, its potential as a potent inhibitor of cytochrome P450 and, therefore, of phase I reactions, mainly aromatic hydroxylation, should be considered when administering it [52]. As a result of the stimulation of these metabolization reactions, piperine improves the bioavailability of drugs that are metabolized in the liver; it increases their plasma half-life, delays their excretion, and increases their therapeutic potential.

8. Potential Application and Future Prospects of Black Pepper as an Oral Iron Supplement

Piperine has the potential to be applied in the formulation of supplements to improve sustained and controlled release bioavailability, to allow a decrease in dosage and dosing frequency, which would increase patient compliance and tolerance [53]. According to what we have described in this manuscript, it could be hypothesized that the thermogenic activity of small amounts of piperine increases the absorption rate of the nutrients administered together with piperine. Therefore, this property enables it to act as a thermonutrient on the epithelial cell membrane of the intestine. However, the features of the thermogenic response modulated by catecholamines are moderately ephemeral. Therefore, the time window of thermogenesis-stimulated absorption in the intestinal tract and the potential increase in bioavailability of piperine-co-administered nutrients is temporarily narrow. Therefore, the activity induced by piperine is limited in time, which makes the simultaneous administration of Fe and Bioperine® necessary.

The frequent use of Fe supplements with BioPerine® would stimulate the possibility of achieving more significant positive effects on some of the parameters involved in the metabolism of Fe, with lower doses, which reduces the risks of Fe overload on the gastrointestinal tract and possible deposits in organs and tissues, and attenuates the potent pro-oxidant effect of Fe. The accumulation of Fe inside

the cells increases their oxidative stress, which translates into a high production of free radicals and reactive oxygen and nitrogen species, which contributes directly to muscle damage. This situation is especially critical in athletes, where the skeletal muscle has been damaged due to physical activity; one could add the pro-oxidant event of excess Fe derived from hyper-supplementation. Therefore, muscle damage will be exacerbated, which directly affects health and physical performance.

However, iron metabolism is not the only player affecting muscle performance. Due to the complexity of muscle physiology, other factors can have a decisive influence on skeletal muscle, such as age, lipotoxicity, muscle stiffness, and the role of caloric restriction in muscle health [54–56]. In this way, sarcopenia is the age-associated loss of muscle mass and function. Aging is a physiological process involving biochemical and morphological changes that are affected throughout the muscle [54]. These biological aging processes include reduced regenerative capacity by stem cell exhaustion, cellular senescence, increased pro-inflammatory intercellular signaling, insulin resistance, lipotoxicity, dysregulated nutrient sensing, mitochondrial dysfunction and increased oxidative stress [54,56]. This damage is associated with inflammation, protein catabolism, myocyte apoptosis, alteration of functional capacity, muscle atrophy, increase muscle stiffness, and, therefore, decreased muscle performance [56].

Moreover, histopathological muscle changes as a result of the pathways triggered by sarcopenia, which affect muscle performance, are changes in muscle fiber size and number, causing mass muscle reduction with age and selective atrophy of muscle fibers [54,56]. In addition, another factor to consider in performance and muscle function is caloric restriction (CR) [55]. CR-induced effects are related to aging in skeletal muscle. In an animal model, Chen et al. [55] observed that CR modulates mTOR signaling and the ubiquitin-proteasome pathway (UPP). CR did not alter mTOR signaling and UPP in the soleus muscles of young and adult rats, whereas CR altered the pathways in middle-aged rats, stimulating muscle protein degradation [55].

Finally, we believe that more studies are necessary because the pharmacokinetics of drugs and natural products is modulated with administered of piperine, which could enhance both therapeutic and adverse effects. For this reason, it is necessary to know the set of interactions between piperine and Fe by studying the effects of variations in dose, dose frequency and duration of treatment on the ability to modify (stimulating or inhibiting) the activity of metabolic enzymes and transporters, depending on the conditions of the treatment administered. For safe application of piperine and iron, clinical studies with long-term interventions should be conducted. This will allow the therapeutic action of piperine to be exploited in formulations with other products. Thus, more research is needed, and this may lead to an advance in oral Fe supplementation for physically active healthy individuals.

9. Conclusions

Supplementation with a natural product, Bioperine®, in the form of black pepper, as a bioavailability enhancer, could have a potential application as an athlete Fe supplement for improving hematological parameters. Therefore, it is proposed as an innovative food therapy without side effects in ID patients supplemented with Fe for the control of Fe status with or without anemia.

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ELSEVIER

Piperine derived from black pepper increases the plasma levels of coenzyme Q10 following oral supplementation

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*An extract from the fruits of black pepper consisting of a minimum of 98% pure piperine was evaluated in a clinical study using a double-blind design. The relative bioavailability of 90 mg and 120 mg of coenzyme Q10 administered in a single-dose experiment or in separate experiments for 14 and 21 days with placebo or with 5 mg of piperine was determined by comparing measured changes in plasma concentration. The inter-subject variability was minimized by limiting the selection of individuals to healthy adult male volunteers with (presupplementation) fasting coenzyme Q10 values between 0.30 and 0.60 mg/L. The results of the single-dose study and the 14-day study indicate smaller, but not significant, increases in plasma concentrations of coenzyme Q10 in the control group compared with the group receiving coenzyme Q10 with a supplement of piperine. Supplementation of 120 mg coenzyme Q10 with piperine for 21 days produced a statistically significant (*p = 0.0348*), approximately 30% greater, area under the plasma curve than was observed during supplementation with coenzyme Q10 plus placebo. It is postulated that the bioenhancing mechanism of piperine to increase plasma levels of supplemental coenzyme Q10 is nonspecific and possibly based on its description in the literature as a thermonutrient.* (J. Nutr. Biochem. 11:109–113, 2000)

Keywords: piper nigrum; black pepper; piperine; bioperine; coenzyme Q10; bioavailability

Introduction

Piperine belongs to a group of compounds known as "vanilloids," because they are distinguished by the presence of a chemical group based on the structure of vanillin. Piperine is a naturally occurring compound present as the major pungent ingredient (1–9%) in various parts of plants from the family *Piperaceae*.[1] Piperine, an alkaloid (1-peperoyl piperidine), has been previously evaluated for its potential to enhance the serum levels of drugs and nutrients in animals and humans.[2–9] Compounds studied include drugs such as vasicine,[2] pyrazinamide,[3] rifampicin,[4] isoniazid,[3] propranolol,[5] theophylline,[5] and phenytoin,[6] and nutrients[8,9] such as fat soluble beta-carotene, water soluble vitamin B_6, vitamin C, and the mineral selenium in the form of L-selenomethionine. The results of clinical studies of piperine with drugs indicate that piperine administered orally at a single dose of 20 to 50 mg may significantly increase serum drug levels by reducing the clearance of drugs, both naturally derived and synthetic. This effect is primarily due to piperine's ability to inhibit (by a noncompetitive mechanism) the xenobiotic (drug) metabolizing enzymes when administered in a high dose.[3,4] On the other hand, the increased nutrient absorption in the presence of piperine appears to be independent of the inhibition of the biotransforming enzymes and has been achieved with as little as 5 mg of piperine coadministered with the supplemented nutrient.[8,9]

The purpose of the this study was to compare the serum response to the biologically important nutritional compound coenzyme Q10, administered to healthy male volunteers with piperine or a placebo under various treatment protocols.

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Materials and methods

Subjects

Twelve healthy adult male subjects were enrolled in this study. Prior to selection, the prospective subjects were instructed regarding the study design and its objectives. All individuals were given an opportunity to ask questions regarding the proposed study, and the volunteers gave their written informed consent. The subjects, aged 20 to 47 years, were in good health and were nonsmokers and nondrinkers of alcohol (less than one alcoholic drink per day was allowed). In addition, they had not taken any type of medication, prescription or over-the-counter, or used any nutritional supplements during the 4 months prior to the study. Inclusion in the study was restricted to individuals who had never ingested coenzyme Q10 supplements and who also provided two consecutive (1 week apart) fasting baseline plasma coenzyme Q10 values between 0.30 and 0.60 mg/L.

All subjects stayed on their self-selected diets. They were instructed not to change their eating habits, particularly with regard to fruit or vegetable consumption, during the supplementation period. From day 0 until day 21, the subjects were provided with a standard breakfast that consisted of 8 oz. of whole milk and a large Danish pastry (15 g fat).

The experimental protocol was reviewed and approved by the Institutional Review Board at Our Lady of Mercy Medical Center (Bronx, NY USA). The study was performed at the Biomedical Research Laboratory of Our Lady of Mercy Medical Center (Bronx, NY USA).

Formulations tested

The control group received a GNC (General Nutrition Center) Pro Performance Brand coenzyme Q10 soft-gel capsule (30 mg of coenzyme Q10 per capsule), plus a two-piece, hard-shell placebo capsule provided by Sabinsa Corp. (Lot # RD/BPP/02; Piscataway, NJ USA). They were administered together in a single dose (90 mg of coenzyme Q10), 14 day supplementation (90 mg of coenzyme Q10 per day) and 21 day supplementation (120 mg of coenzyme Q10 per day). The active group received coenzyme Q10 soft-gel capsules and a two-piece, hard-shell capsule containing 5 mg piperine provided by Sabinsa Corp. (Lot # RD/BPC/02). A specific brand of piperine, Bioperine®, was used in the study. Bioperine is composed of a minimum of 98% pure alkaloid piperine extracted from the fruits of black pepper (*Piper nigrum Linn*).[8,9]

Experimental design

The subjects were randomly assigned into two groups of six subjects each. After assignment, each volunteer arrived at the laboratory between 6:30 and 7:30 AM following an overnight fast on study days −7 and 0. On both occasions, a fasting blood sample was collected from each subject for determination of two baseline plasma coenzyme Q10 measurements. Immediately following the collection of the second baseline blood sample (day 0), each volunteer was started on a particular experimental dosing regimen. The ingestion of the supplement on an empty stomach was fully supervised (in the presence of an appointed supervisor), and 30 minutes later the standardized breakfast was eaten (8 oz. of whole milk and a large Danish pastry). In the single-dose study, fasting blood samples were collected for coenzyme Q10 analysis at intervals of 2, 4, 5, 6, 7, and 8 hours; in the 14-day study, fasting blood samples were collected for plasma coenzyme Q10 analysis on days 2, 4, 7, 10, and 14; and in the 21 day study, fasting blood samples were collected for plasma coenzyme Q10 analysis on days 4, 7, 10, 14, and 21.

Table 1 Hourly changes in coenzyme Q10 plasma values following a single dose administration of 90 mg coenzyme Q10 either with placebo or with 5 mg piperine in 12 volunteers

	Mean increase in plasma coenzyme Q10 levels (mg/L)	
Time (hr)	Q10 + placebo group	Q10 + piperine group
0	0.435 ± 0.145	0.413 ± 0.117
2	0.598 ± 0.138	0.576 ± 0.11
4	0.720 ± 0.111	0.741 ± 0.083
5	0.788 ± 0.091	0.825 ± 0.125
6	0.840 ± 0.106	0.846 + 0.143
7	0.853 ± 0.208	0.808 ± 0.2
8	0.763 ± 0.119	0.733 ± 0.107

Sample collection and analysis

Blood samples were collected in 7 mL vacutainer tubes that contained ethylendaimine-tetraacetic acid (EDTA) for the anticoagulant and placed in a test tube rack that was protected from heat and light. Plasma was separated by centrifugation (4°C, 15 min, 1,200 × g) within 60 minutes of collection. Immediately following centrifugation, plasma was transferred to 2 mL cryogenic vials and stored at −85°C until analysis. Coenzyme Q10 analyses were completed within 3 weeks of collection by reversed-phase high performance liquid chromatography (HPLC).[10]

The area under the plasma concentration curve (AUC) of coenzyme Q10 was calculated as follows:

$$\sum_{i=1}^{5} (t_i - t_{i-1}) \left[\frac{(v_i + v_{i-1})}{2} - v_0 \right]$$

where t refers to the day following initiation of supplementation, I refers to the specific sampling period, and v equals the plasma coenzyme Q10 level at the t_i period. The variable v_o at period one is the arithmetic mean of two baseline measurements.

Statistical data analysis

Analysis of variance and *t*-tests were used to test the null hypothesis that the means of the baseline coenzyme Q10 values were equal across the two groups, that the mean absolute (and relative) change in plasma coenzyme Q10 values between baseline and each data point during the ingestion period was equal for the two groups, and that the mean area under the plasma coenzyme Q10 curve was equal for the two formulations.[11]

Results

Hourly changes in coenzyme Q10 plasma values following single-dose administration of 90 mg coenzyme Q10 with placebo or with 5 mg piperine

The single-dose administration of 90 mg coenzyme Q10 with placebo or with piperine indicated a wide variation in the plasma coenzyme Q10 levels in the 12 subjects. The numerically higher net plasma increases (mg/L) were found in the group receiving coenzyme Q10 with piperine rather than in the group receiving coenzyme Q10 with placebo (*Table 1*). In addition, the Tmax for the coenzyme Q10 and piperine group was attained 1 hour sooner than the Tmax for the control group (6 versus 7 hours; *Figure 1*). However, the differences between the two dosing groups were not statistically significant.

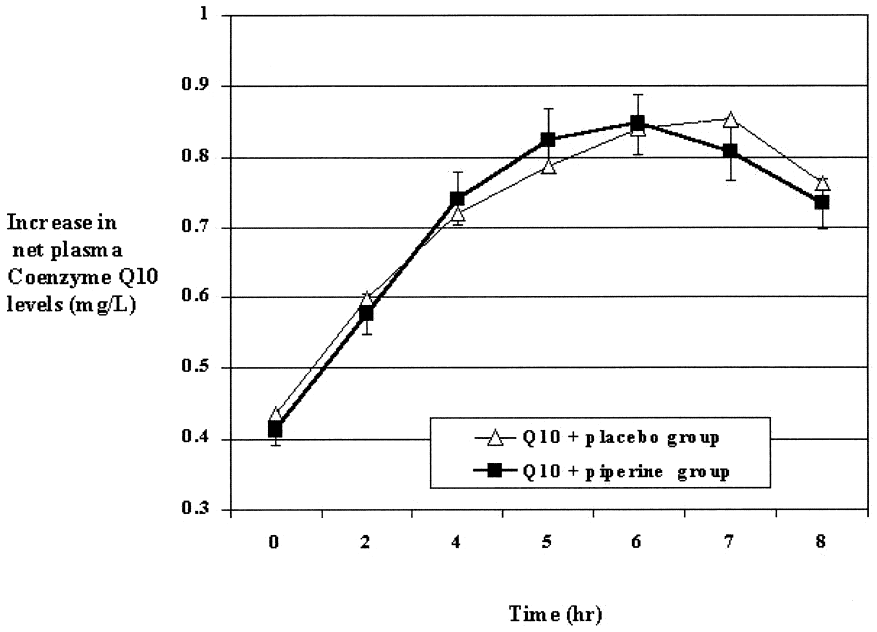

Figure 1 Hourly changes in coenzyme Q10 plasma values following single dose administration of 90 mg coenzyme Q10 either with placebo or with 5 mg piperine in 12 volunteers.

Absolute changes in plasma coenzyme Q10 from baseline to day 14

The mean absolute change (change from baseline to post-supplementation value) in plasma coenzyme Q10 values and the mean AUC from baseline to day 14 were numerically greater for the active group (90 mg coenzyme Q10 plus 5 mg piperine per day) than for the placebo group (daily 90 mg coenzyme Q10 with placebo). The mean baseline coenzyme Q10 value was 0.44 ± 0.09 mg/L for the active group and 0.46 ± 0.13 mg/L for the placebo group. The mean absolute change after 14 days of supplementation was 0.21 ± 0.04 mg/L and 0.19 ± 0.07 mg/L, respectively; and the mean AUC was 1.098 ± 0.439 and 1.027 ± 0.441 (mg/L) × (days), respectively. However, changes in the active group were not significantly different from the values determined for the control group.

Absolute changes in plasma coenzyme Q10 from baseline to day 21

The mean absolute change in plasma coenzyme Q10 values and the mean AUC from baseline to day 21 were significantly greater for the active study than for the placebo study (*Table 2*).

Coadministration of 5 mg piperine with 120 mg coenzyme Q10 daily resulted in a statistically significant ($p = 0.0369$) absolute increase in plasma levels of coenzyme Q10 of 1.12 mg/L compared with the absolute increase of 0.85 mg/L in the control group (approximately 32% greater increase in active versus control group; *Table 2*). Corresponding values measured as the area under the plasma coenzyme Q10 curve in the active group receiving piperine were higher and statistically significant ($p = 0.0348$) when compared with the control group (15.38 and 11.81 mg/L; approximately 30% greater increase in active versus control group; *Table 2*).

Discussion

This study is one in a series of clinical trials conducted on the alkaloid piperine to explore the use of this compound and the mechanism(s) by which it enhances the gastrointestinal absorption of nutrients. The selected nutrient, coenzyme Q10, is different from previously studied vitamins and minerals because the body synthesizes it. The increased plasma response to supplemental coenzyme Q10 is of clinical significance because its deficiency has been found in patients with breast cancer and nonmalignant breast

Table 2 Absolute change in plasma coenzyme Q10 from baseline to day 21 of daily ingestion of 120 mg coenzyme Q10 with placebo or with a concurrent dose of 5 mg piperine in 12 volunteers

Treatment	Mean baseline value ± SD coenzyme Q10 mg/L	Mean absolute change ± SD coenzyme Q10 mg/L	Mean area under the curve (AUC) (mg/L)x (days)
Coenzyme Q10 + placebo	0.50 ± 0.09	0.85 ± 0.20*	11.81 ± 2.66†
Coenzyme Q10 + piperine	0.49 ± 0.09	1.12 ± 0.20*	15.38 ± 2.40†

* Difference between mean absolute change in placebo and active group statistically significant ($p = 0.0369$).
† Difference between mean absolute change in placebo and active group statistically significant ($p = 0.0348$).

tumors.[12] Low levels of coenzyme Q10 have been found in patients with insulin dependent diabetes mellitus,[13] patients with severe ischemic heart disease,[14] and endurance trained individuals.[14] Supplementation with coenzyme Q10 has been shown to be beneficial for the treatment of congestive heart failure,[15] dyslipidemia,[16] complications of diabetes,[17] Parkinson's syndrome[18] and as a potential therapy for some forms of male infertility.[19]

The normal plasma levels of coenzyme Q10 reported in literature vary. According to one report, it is between 0.36 and 0.8 mg/L for men,[20] whereas a range of 0.46 to 0.57 mg/L for men and women is reported by other researchers.[21] The levels of coenzyme Q10 in different age groups were reported as follows: <30 years, 0.42 ± 0.16 mg/L; 30 to 49 years, 0.48 ± 0.22 mg/L; >50 years, 0.47 ± 0.10 mg/L.[21] The breakdown of normal coenzyme Q10 values based on gender showed 0.49 ± 0.21 mg/L plasma levels in men and 0.44 ± 0.15 mg/L plasma levels in women. Supplemental coenzyme Q10 is poorly absorbed from the digestive tract and is slowly eliminated from plasma, with a long plasma half-life of 33.19 ± 5.32 hours.[22]

The current study tested three dosing regimens of coenzyme Q10 with and without an additional supplement of 5 mg piperine: a single-dose study evaluating the absorption rate of 90 mg coenzyme Q10 within 8 hours, 14-day administration of 90 mg coenzyme Q10 per day, and 21-day administration of 120 mg of coenzyme Q10 per day. The inclusion criterium for participants in this study was a plasma level of coenzyme Q10 of 0.30 to 0.60 mg/L. To further minimize the inter-subject variability, this initial evaluation of the effects of piperine on coenzyme Q10 bioavailability was carried out selectively with healthy, young male volunteers only.

The plasma response to coenzyme Q10 administered in a single dose, as well as in the course of 14-day supplementation, resulted in a wide variation of coenzyme Q10 plasma levels in the control and piperine subjects. The numerically higher net plasma levels of coenzyme Q10 obtained in both these experiments within the piperine groups were not significantly higher than those in the respective control groups. Nevertheless, the results from these experiments suggest that piperine may exert a bioenhancing effect on coenzyme Q10 gastrointestinal absorption.

The third experiment involving the coadministration of 5 mg piperine with 120 mg coenzyme Q10 once daily for 21 days showed significant increase in the plasma levels of coenzyme Q10 in comparison to the control group. The increase in the AUC was 30%, whereas the absolute change in the plasma values was 32%.

Our results suggest that piperine supplementation may result in improved absorption of coenzyme Q10 in healthy subjects. It also appears that a longer period of coadministration of piperine with 120 mg rather than 90 mg of coenzyme Q10 per day may result in significantly better absorption of coenzyme Q10 as measured by its plasma levels.

The mechanism(s) by which piperine increases the absorption of coenzyme Q10 is likely nonspecific and operates directly in the gastrointestinal tract. These mechanisms, based on literature data, may involve increased gastrointestinal blood supply,[23] increased micelle formation,[23] and epithelial cell wall modification due to the lipophilic nature of the compound.[24] The most interesting mode of action of piperine may be due to its postulated thermogenic[25] properties and the increase in bioenergetic processes[26] in the gastrointestinal epithelium described in our previously published study on its *thermonutrient* activity.[8]

This preliminary report on increased gastrointestinal absorption of the coenzyme Q10 with piperine presents an interesting concept to be further evaluated. The combined supplementation of coenzyme Q10 with piperine may be a practical approach to enhance this nutrient bioavailability, especially in patients diagnosed with low plasma levels of coenzyme Q10 and in elderly individuals with poor gastrointestinal absorption.

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Influence of Piperine on the Pharmacokinetics of Curcumin in Animals and Human Volunteers

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Abstract: **The medicinal properties of curcumin obtained from *Curcuma longa* L. cannot be utilised because of poor bioavailability due to its rapid metabolism in the liver and intestinal wall. In this study, the effect of combining piperine, a known inhibitor of hepatic and intestinal glucuronidation, was evaluated on the bioavailability of curcumin in rats and healthy human volunteers. When curcumin was given alone, in the dose 2 g/kg to rats, moderate serum concentrations were achieved over a period of 4 h. Concomitant administration of piperine 20 mg/kg increased the serum concentration of curcumin for a short period of 1–2 h post drug. Time to maximum was significantly increased ($P < 0.02$) while elimination half life and clearance significantly decreased ($P < 0.02$), and the bioavailability was increased by 154 %. On the other hand in humans after a dose of 2 g curcumin alone, serum levels were either undetectable or very low. Concomitant administration of piperine 20 mg produced much higher concentrations from 0.25 to 1 h post drug ($P < 0.01$ at 0.25 and 0.5 h; $P < 0.001$ at 1 h), the increase in bioavailability was 2000 %. The study shows that in the dosages used, piperine enhances the serum concentration, extent of absorption and bioavailability of curcumin in both rats and humans with no adverse effects.**

Key words: **Curcumin, piperine, pharmacokinetics, *Curcuma longa*, Zingiberaceae.**

Introduction

Curcumin is obtained from *Curcuma longa* L. (Zingiberaceae), a perennial herb widely cultivated in tropical regions of Asia. Its rhizome is extensively used for imparting colour and flavour to food. Current traditional Indian medicine claims the use of its powder, turmeric, against a wide variety of diseases (1). Extensive scientific research (2) on curcumin has demonstrated a wide spectrum of therapeutic effects which range from anti-inflammatory, wound healing, antispasmodic, anticoagulant, antitumor activities (3) and recently, with potential utility in autoimmune deficiency syndrome (4).

Pharmacokinetic properties of curcumin indicate that following oral administration, it is poorly absorbed (3) and only traces of the compound appear in the blood, while most of it is excreted in the faeces (5). The transformation of curcumin into an unidentified compound during absorption (6) and its glucuronidation in the liver (5, 7) are probably responsible for its low concentration in blood.

Black pepper (*Piper nigrum* L.) and long pepper (*Piper longum* L.) have been in use as spices from ancient times throughout the world. A major component of the *Piper* species is the alkaloid piperine (1-piperoylpiperidine), which has been reported to enhance the bioavailability of drugs by inhibition of glucuronidation in the liver (8) and small intestine (9).

In view of the potential therapeutic utility of curcumin it appeared pertinent to examine the effect of piperine, a known hepatic and intestinal metabolic inhibitor, on the pharmacokinetic disposition of curcumin in animals and man, to provide a scientific rationale for assigning it a rightful place in the pharmacologists armamentarium.

Materials and Methods

Animal studies

Albino Wistar rats (n = 96) of both sexes (150–200 g) were chosen for the study. They were housed in well ventilated cages, fed on commercial rat pellets supplied by Hind Lever, Mumbai, with tap water ad libitum. They were divided into two sex and weight matched groups (n = 6/group/time cut), one group for administration of curcumin and the other for concomitant curcumin and piperine. Curcumin and piperine were supplied in pure powder form by Sami Chemicals and Extracts, Bangalore, India. Both the compounds were administered orally to fasted rats as an aqueous suspension. In the group that received both drugs, curcumin was administered first followed immediately by piperine. Control rats received water only. Curcumin was given in a dose of 2 g/kg and piperine, 20 mg/kg.

Under ether anaesthesia, pre and post drug jugular vein blood samples were collected from both groups of rats into centrifuge tubes at the following time intervals – 0, 0.25, 0.50, 0.75, 1, 2, 3, 4, 5, and 6 h. The blood was allowed to clot at room temperature for about 1 h and then centrifuged at 3000 rpm for 10 min. The serum was separated out carefully using Pasteur pipettes into storage tubes and frozen at – 20 °C prior to analysis.

Planta Medica 64 (1998) 353–356

Human volunteer studies

Ten healthy male volunteers, 20 to 26 years, weighing 50–75 kg (mean 60 ± 1.93) participated in a randomized cross over trial, to determine the comparative bioavailability and pharmacokinetic profile of curcumin when given alone and with piperine. Complete physical examination and an electrocardiogram were done. Laboratory tests comprising complete blood counts and haemoglobin percentage, blood biochemistry consisting of blood urea nitrogen (BUN), serum creatinine, total and conjugated bilirubin, alkaline phosphatase, aspartate transaminase (ASAT), alanine transaminase (ALAT), urine albumin, and sugar were performed to confirm that the subjects included in the study were normal. The study was formally approved by the Institutional Ethical Committee and informed consent was obtained from all subjects.

Subjects abstained from food since 10 pm of the previous evening and reported to the laboratory at 7.00 am. Venepuncture was done using a 20 g scalp vein set with heparin lock and left *in situ*. Blood samples (5 ml) were collected (without anticoagulant) at 0, 0.25, 0.50, 0.75, 1, 2, 3, 4, 5, and 6 h post drug. Blood was allowed to clot at room temperature for 1 h. Serum separation and storage until analysis was as explained earlier. Following basal blood sample collection 2 g of pure curcumin powder (4 capsules of 500 mg each) or 2 g of pure curcumin powder combined with 20 mg of pure piperine powder (4 capsules of 500 mg curcumin + 5 mg piperine each; identical capsules prepared by Sami Chemicals and Extracts, Bangalore, India) was given with 150 ml of water. Blood sampling after curcumin *per se* and curcumin with piperine was done on two occasions, separated by a two week wash out period on the same volunteers. The following precautions were taken during the trial: subjects refrained from smoking, consuming alcohol or beverages, and from taking drugs of any kind 24 hours prior to and during the trial. Standard meals were given to all the participants on the day of the test.

Analytical methods

Estimation of curcumin was done by reverse phase high pressure liquid chromatography (HPLC) using a modification of the method described by Tannesen et al. (10). The modification was done by Sami Chemicals and Extracts, Bangalore, India, and is detailed below: the mobile phase used was ethanol : methanol (60 : 40) instead of only ethanol and the flow rate was changed from 1.2 ml/min to 1 ml/min. HPLC grade methanol and low actinic glassware protected from light were used for the entire procedure.

Extraction and preparation of standard solution

Curcumin (25 mg) was dissolved and diluted to 25 ml with methanol in a volumetric flask; 0.1 ml (100 µl) of this was transferred to a volumetric flask and diluted with methanol upto 10 ml making a 10 ppm solution; 0.1 ml of this 10 ppm solution was transferred to another 10 ml volumetric flask and the volume made up with methanol making a 0.1 ppm solution.

Extraction of curcumin from serum and preparation of sample

Serum samples stored at −20 °C were equilibated to room temperature before analysis. A portion of 1 ml was transferred into a 10 ml volumetric flask and about 5 ml of methanol added. The mixture was shaken thoroughly and heated at 80 °C on a water bath for half an hour. After cooling to room temperature, methanol was added to make up the volume to 10 ml and mixed well. The turbid solution was transferred into a 15 ml centrifuge tube and centrifuged at 4000 RPM for 10 minutes. The supernatant was collected by means of a 25 ml syringe and 10 cm needle (Luer lock) and the clear solution filtered through a 0.45 µm, 13 mm millipore membrane filter, into a narrow end test tube. 20 µl of the solution were injected into the chromatograph for carrying out the HPLC analysis.

Samples were read by UV absorbance of 254 nM. The recovery rate experiments were carried out by adding a known amount of standard curcumin to the serum and the added curcumin extracted as per procedure and quantified. The recovery rate of curcumin from serum ranged from 87–89.9 %. The minimum level of detection of curcumin was 0.001 µg/ml.

Calculation

$$\text{Content of curcumin in } \mu g/ml \text{ in the test sample} = \frac{\text{Standard Reading} \times \text{standard concentration}}{\text{Standard Reading} \times \text{sample concentration}}$$

Treatment of pharmacokinetic data

For calculation of pharmacokinetic parameters (PK), curve fitting was carried out by a model independent method with non-linear least-square regression analysis using a computer designed programme "PHARMKIT". This programme uses an algorithm called "SIMPLEX" for calculating non-linear least squares. The various PK parameters calculated were: absorption half life ($t^1/_{2(a)}$), elimination half live ($t^1/_{2el}$), volume of distribution (Vd); and clearence (Cl). Areas under the concentration time curve ($AUC_{0\text{-}tn}$) was calculated using the trapezoidal method. Maximum concentration (C_{max}) and time to max (T_{max}) are the observed values. Relative bioavailability (F) was calculated using the formula:

$$F = \frac{\text{AUC Curcumin + Piperine}}{\text{AUC Curcumin}} \times 100$$

Statistical analysis

Serum concentration time curves and the PK parameters from animal data were analysed using the Student's "t" test, while the paired "t" test was used for comparing serum concentration curves in humans. PK parameters of curcumin when given alone in humans could not be calculated as curcumin could not be detected in most of the samples.

Results

Animal studies

Curcumin alone at 2 g/kg, or when combined with piperine, 20 mg/kg, was well tolerated by the rats as they showed no untoward effects for 48 h. Yellow coloured faecal pellets appeared at 30 h postdrug and continued upto 48 h. Perusal of Figure **1** indicates that when curcumin was given alone, peak serum concentrations of 1.00 ± 0.26 µg/ml were attained rapidly within 0.75 h and plateaued till 1 h. Thereafter, the levels declined gradually reaching zero at 5 h. The plasma

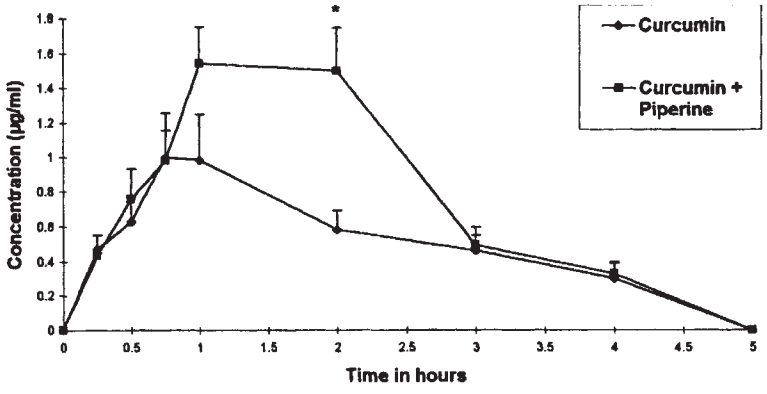

Fig. 1 Serum concentrations µg/ml (mean ± SEM) of curcumin 20 g/kg oral alone and with piperine 20 mg/kg in rats (n = 6/group/time cut). Significance as compared to curcumin alone; *P < 0.02.

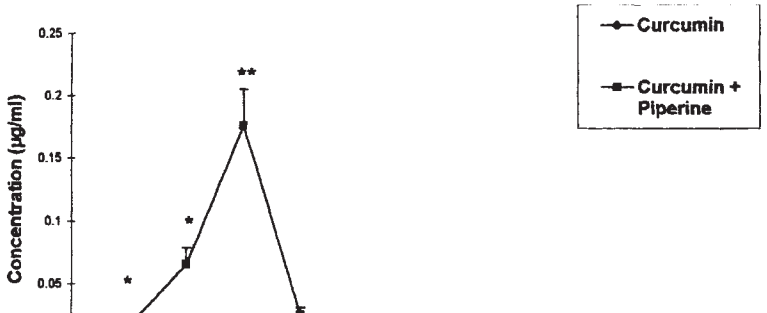

Fig. 2 Serum concentration µg/ml (mean ± SEM) of curcumin 2 g oral alone and with piperine 20 mg in humans (n = 8). Significance as compared to curcumin alone; * P < 0.01; ** P < 0.001.

concentration time curve of curcumin in combination with piperine followed a similar pattern from 0 to 0.75 h and 3 to 5 h. However, piperine produced higher serum concentrations of curcumin at 1 and 2 h (1.55 ± 0.21 and 1.50 ± 0.25 µg/ml) respectively, being significantly higher (P < 0.02) at 2 h. Thus piperine significantly enhanced the serum concentration of curcumin, albeit for a limited duration (although serum samples were collected upto 6 h, values are depicted till 5 h only, since the 6 h value was also "0" in all animals).

Table **1** shows the values (mean ± SEM) of the pharmacokinetic parameters of curcumin *per se* and when combined with piperine. C_{max} was increased from 1.35 ± 0.23 to 1.80 ± 0.16 µg/ml, but was not statisticallyy significant, while T_{max} was significantly increased from 0.83 ± 0.05 to 1.29 ± 0.23 h (P < 0.02). The $t^1/_{2(el)}$ significantly decreased from 1.70 ± 0.58 to 1.05 ± 0.18 h (P < 0.02). Though $t^1/_{2(a)}$ increased from 0.31 ± 0.07 to 0.47 ± 0.03 h and AUC increased from 2.36 ± 0.28 to 3.64 ± 0.31 µg/h/ml, these increases were not statistically significant. Cl significantly decreased from 713.00 ± 12.00 to 495.00 ± 37.00 L/h (P < 0.02), but the decrease in the Vd from 1366.00 ± 248.70 to 782.60 ± 193.90 L/kg was not significant. The relative bioavailability of curcumin when combined with piperine is 154 %.

Human volunteer studies

Curcumin alone or when combined with piperine was well tolerated by all the subjects and there were no adverse or untoward reactions; 2 subjects dropped out of the study for non-medical reasons. Therefore all calculations presented here are based on the data obtained from 8 subjects. In Figure **2** is shown the serum concentration of curcumin *per se* and when given with piperine. Although serum samples were collected upto 6 h, we have depicted values till 3 h, since the 4, 5, and 6 h values were also "0" in all subjects.

Serum levels of curcumin when given alone were either very low or undetectable at most time points in most subjects, explaining the almost flat serum concentration curve (Fig. **2**). However, when piperine was added the serum concentrations of curcumin were significantly increased at the time points

Table 1 Pharmacokinetic parameters (mean ± SEM) of oral curcumin 2 g/kg alone and in combination with piperine 20 mg/kg in rats (n = 6/drug/time cut).

Parameters	Curcumin alone 2 g/kg	Curcumin + Piperine 2 g/kg + 20 mg/kg
C_{max} (µg/ml)	1.35 ± 0.23	1.80 ± 0.16
T_{max} (h)	0.83 ± 0.05	1.29 ± 0.23*
$t^1/_{2(a)}$ (h)	0.31 ± 0.07	0.47 ± 0.03
$t^1/_{2(el)}$ (h)	1.70 ± 0.58	1.05 ± 0.18*
AUC(0–tn) (µg/h/ml)	2.36 ± 0.28	3.64 ± 0.31
Vd (L/kg)	1366.00 ± 248.70	782.90 ± 193.90
Cl (L/h)	713.00 ± 12.00	495.90 ± 37.08*

* P < 0.02: Statistical significance by Students "t"-test.
C_{max}: Maximum serum concentration.
T_{max}: Time to reach maximal serum concentration.
$t^1/_{2(a)}$: Absorption half-life.
$t^1/_{2(el)}$: Elimination half-life.
$AUC_{(0-tn)}$: Area under the concentration time curve.
Vd: Volume of distribution.
Cl: Total clearance.

upto 0.75 h; $P < 0.01$ at 0.25 h and 0.5 h; $P < 0.001$ and at 0.75 h. Subsequently there was a rapid decline upto 1 h and thereafter a gradual decline to zero by 3 h.

In Table **2** are depicted the PK parameters (Mean ± SEM) of curcumin when given alone and with piperine. C_{max} (observed values) when curcumin was given alone was only 0.006 ± 0.005 μg/ml at 1 h whereas when piperine was added the C_{max} (observed value) was increased to 0.18 ± 0.16 μg/ml and was attained earlier, i.e. at 0.75 h. Vd and Cl could not be calculated with curcumin alone as serum levels were not detected at most time points in most subjects. The mean $AUC_{(0-tn)}$, however, was calculated using the trapezoidal method and was found to be 0.004 μg/h/ml, the relative bioavailability of curcumin when given with piperine was therefore 2000 %.

Table 2 Pharmacokinetic parameters (mean ± SEM) of oral curcumin 2 g/kg alone in combination with piperine 20 mg/kg in normal healthy volunteers (n = 8).

Parameters	Curcumin alone 2 g/kg	Curcumin + Piperine 2 g/kg + 20 mg/kg
C_{max} (μg/ml)	0.006 ± 0.005	0.18 ± 0.03
T_{max} (h)	1	0.69 ± 0.07
$t^1/_{2(a)}$ (h)	*	0.11 ± 0.02
$t^1/_{2(el)}$ (h)	*	0.41 ± 0.17
$AUC_{(0-tn)}$ (μg/h/ml)	0.004	0.08 ± 0.01
Vd (L/kg)	*	202.60 ± 78.94
Cl (L/h)	*	7.33 ± 1.25
F (Relative bioavailability)		2000 %

C_{max}: Maximum serum concentration.
T_{max}: Time to reach maximal serum concentration.
$t^1/_{2(a)}$: Absorption half-life.
$t^1/_{2(el)}$: Elimination half-life.
$AUC_{(0-tn)}$: Area under the concentration time curve.
Vd: Volume of distribution.
Cl: Total clearance.
* Not calculated – see text.

Discussion

The results obtained in the study demonstrate that piperine enhances the oral bioavailability of curcumin in both rats and humans at doses that were devoid of adverse side effects. However, certain differences between rat and human with respect to curcumin were evident. Curcumin *per se* attained overall moderate serum concentrations over a 4 h period in rats with peak levels occurring between 0.75 h to 1 h. On the other hand, in humans when curcumin was given alone only negligible serum concentrations of curcumin were detectable the serum concentration-time curve being almost flat. This difference may be due to the high oral dose employed in the rat (2 g/kg), whereas the human dose was about 60 times less, approximately 33 mg/kg. Curcumin serum concentrations reached zero at 5 h in rats and 3 h in humans. Further in rats with the addition of piperine, curcumin achieved higher concentrations than in humans albeit for a short period, took a longer time to peak and declined slowly. Whereas in humans T_{max} was attained earlier and then declined rapidly. This rapidity in decline is more apparent probably because of the higher levels of curcumin achieved with piperine as compared to curcumin alone. There was an increase in the AUC though not significant and an increase in bioavailability of curcumin by about one and a half times as compared to curcumin given alone in both rats and humans. In rats when piperine was added to curcumin both Vd and Cl decreased which may have also contributed to the higher concentration, such a comparison was not possible in humans for reasons explained earlier. Our findings concerning absorption of curcumin in rats are in agreement with data obtained by Wahlstrom and Blennow (11), who showed that when Sprague Dawley rats were given curcumin 1 g/kg *p.o.*, measurement of blood plasma levels and biliary excretion indicated some absorption from the gut with no apparent toxic effects upto 5 g/kg *p.o.* Likewise, Khanna et al. (12) found that after curcumin, 100 mg/kg *p.o.*, 74 % was absorbed from the gastrointestinal tract within the first 5 h, while complete elimination occurred within 48 h. Our results are, however, in conflict with studies by Ravindranath and Chandrasekhara (6), who could not detect curcumin in portal or heart blood samples upto 24 h in rats given curcumin 400 mg/kg *p.o.* They, however, did report 60 % absorption of curcumin as determined by the amount excreted in the faeces.

There is evidence that piperine is a potent inhibitor of drug metabolism, and glucuronidation altering the disposition and bioavailability of a large number of drugs (8). Further piperine at 20 mg in humans has also been shown to produce earlier T_{max}, higher C_{max} and AUC of drugs like propranolol and theophylline (13). This property of piperine suggests that it may be involved in inhibiting the metabolism of curcumin and enhancing its bioavailability.

In conclusion, the study shows that piperine enhances the serum concentration and bioavailability of curcumin in rats and man probably due to increased absorption and reduced metabolism.

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REVIEW ARTICLE

Piperine: Old Spice and New Nutraceutical?

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Abstract: ***Background*:** Many of the activities associated with pepper fruits have been attributed to piperine, the most active compound present in these spices.

***Objective*:** This paper aims to provide an overview of the known properties of piperine, *i.e.* piperine's chemistry, its physiological activity, documented interactions as a bioenhancer and reported data concerning its toxicity, antioxidant properties and anticancer activity.

***Discussion*:** It is known that piperine possesses several properties. In its interaction with other drugs, it can act as a bioavailability enhancer; this effect is also manifested in combination with other nutraceuticals, *e.g.* with curcumin, *i.e.* piperine can modify curcumin's antioxidant, anti-inflammatory, antimicrobial and anticancer effects. Piperine displays significant immunomodulating, antioxidant, chemopreventive and anticancer activity; these effects have been shown to be dose-dependent and tissue-specific. However, the main limitation associated with piperine seems to be its low bioavailability, a disadvantage that innovative formulations are overcoming.

***Conclusion*:** It is predicted that an increasing number of studies will focus on piperine, especially those directed towards unraveling its properties at molecular level. The current knowledge about the action of piperine will form a foundation for ways to improve piperine's bioavailability *e.g.* exploitation of different carrier systems. The therapeutical applications of this compound will be clarified, and piperine will be recognized as an important nutraceutical.

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1. INTRODUCTION

1.1. Nutraceuticals: An Overview

The link between diet and health has been known since ancient times. Hippocrates wrote about 2500 years ago "*Let food be thy medicine and medicine be thy food*"; this continues to be relevant today [1]. However, with the advancement of food science and nutrition, the role of diet has changed from simply preventing nutritional deficiencies in promoting optimal well-being and improving the quality of human life [2].

For example, the prolongation in lifespan has increased interest in the role of diet in the prevention of age- related chronic diseases as well as reducing healthcare costs [3].

In the mid-1980s, the concept of functional foods was developed [4]. These are foods which when consumed as a part of a regular diet, may exert beneficial effects, *e.g.* they may act on one or more targets or functions [5]. Similarly, some types of unmodified whole, processed, enhanced or fortified food can be considered as functional foods [6]. Several years later, Dr. De Felice introduced the term "*nutraceutical*" as "*a food or part of a food that provides medicinal and health benefits, including the prevention and/or treatment of a disease*" [7]. At present, in the absence of clear and universally accepted definitions, there is an overlap between nutraceuticals and functional food, and this passes over to other health-related food products [8]. Therefore, in addition to nutraceuticals, dietary supplements can also be viewed as functional [9]. These products represent concentrated forms of natural bioactive substances in a suitable pharmaceutical form but they are used in dosages that exceed those that can be obtained from a regular diet [10].

In addition to nutrients such as vitamins, minerals, amino acids, peptides, and some fatty acids, there are other specific bioactive compounds that can be considered as nutraceuticals: enzymes, pre- and probiotics, coenzyme Q10, glucosamine, chondroitin, etc., as well as many phytochemicals (polyphenols, sulfur-containing compounds, alkaloids, terpenoids etc.) [11, 12]. There are several ways to classify nutraceuticals *i.e.* based on the bioactive compounds present [3, 13, 14] and their functions [3, 9]; according to their origin [1, 3, 8, 9]; their concentrated sources [1, 9]; mechanisms of actions and health benefits [1, 3, 14, 15] or their chemical nature [1, 9, 15]. Nutraceutical classifications can also be based on nutraceutical bioaccessibility, absorption, or chemical transformation within the gastrointestinal tract, as these can be factors limiting their oral bioavailability [16]. Additionally, nutraceuticals can be classified into potential and/or established nutraceuticals according to their proven beneficial effects as demonstrated in either preclinical or clinical studies [17]. At present, only probiotics, prebiotics, omega-3 acids, and antioxidants have been placed in the category of established nutraceuticals [18]. Therefore, it is evident that problems are encountered in classifying nutraceuticals, *i.e.* one substance can be placed into one or more categories. Table **1** lists several nutraceuticals categorized according to their chemical and biological properties [11, 13, 14, 19].

In addition to their sensory properties, the herbs and spices in our diet are rich sources of different phytochemicals with putative beneficial effects *e.g.* antioxidative, anti-inflammatory, chemopreventive, antimutagenic, immune-modulatory properties [20-22].

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Table 1. Classification of nutraceuticals.

Nutraceuticals			
According to Chemical Composition			**According to Effects Against a Specific Disease**
Nutrients (chemical substances with established nutritional functions)	Herbs* (herbs and herb parts as concentrates, extracts…)	Dietary supplements (products - tablets, capsules, liquids, powders, *etc*., administered orally to enrich the diet)	Cardiovascular protective agents Antidiabetic agents Antiobesity agents Anticancer agents Antidepressive agents Agents that improve vision Agents with effects on osteoarthritis and other conditions affecting the musculoskeletal system
Vitamin A Complex B vitamins (B_1, B_2, B_3, B_6) Vitamin C Vitamin K Folic acid Minerals (Ca, Fe, Mg, P, Cr, Co, Cu, Zn, I) Amino acids Fatty acids	Aloe (*Aloe vera L.*) Garlic (*Allium sativum L.*) Curcumin (*Curcuma longa*) Green tea (*Camelia sinensis)* Echinacea (*Echinacea purpurea L.*) Ginkgo (*Ginkgo biloba L.*) St. John's wort (*Hypericum perforatum L.*) Chamomile (*Matricaria recutita L.*) Melissa (*Melissa officinalis L.*) Ginseng (*Panax ginseng*) Black pepper (*Piper nigrum*) Valerian (*Valeriana oficinalis L.*) Ginger (*Zingiber officinale Rosc.*)	Ingredients incorporated into ketogenic diets (*e.g*. coenzyme Q10) Dietary fibers Phytoestrogens Different species of edible mushrooms (mushroom beta- glucans or mushroom polysaccharides) Glucosamine sulfate Chondroitin sulfate Peptides and hydrolysates Probiotics Carotenoids (*e.g*. lycopene, lutein) Unsaturated fatty acids (*e.g*. EPA, DHA, CLA)	

*Selection has been based on the most frequent use (adapted with modifications from [11, 13, 14, 19]).

Due to their potent biological activities and low or non- existent side effects, some of these phytochemicals are being investigated in preclinical and clinical trials as a new approach to treating diseases [23, 24] or as lead compounds in the design of innovative drugs [25]. Moreover, there is evidence that some phytochemicals can enhance the bioefficacy of drugs like antimicrobials, antihypertensives, anticancer drugs, and improve the bioavailability of some nutraceuticals [26, 27]. Due to their natural origin, ease of access, cost-effectiveness and satisfactory safety profiles, these kinds of nutraceuticals have attracted the attention of scientists, consumers, and commercial enterprises [28].

1.2. Piperine as a Nutraceutical

Piperine (E,E)-1-piperoylpiperidine or (E,E)-1-[5-(1,3-benzodioxol-5-yl)-1-oxo-2,4 pentdienyl]-piperidine is an alkaloid present in the piper species, the fruit of black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.). Piperine has been used in traditional medicine for a long time, in treatment of various conditions: rheumatism and muscular aches, as a digestive tonic, in dyspepsia, in flatulence and indigestion, as antipyretic, for throat pain and cough; as antiseptic, bactericide, insecticide, diuretic, *etc*. [29]. In addition to its traditional use, crucial non-traditional nutraceutical effects have been proposed for piperine, based on cell-based studies, animal studies and human studies, reviewed in several articles [26, 29-36]. For example, piperine increases the bioavailability of several drugs from 30 up to 200 fold; for curcumin, this enhancing ability was almost 9-10 fold [37, 38].

Piperine is a molecule that is a promising nutraceutical, with some of its potential uses represented in Fig. (**1**). Moreover, its structure is especially suitable for chemical modifications and alterations, it can present an advantage in the development of novel compounds with various health protective activities.

2. CHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF PIPERINE

2.1. Chemistry of Piperine and its Importance for the Compound's Mechanism of Action

The piperine type of amides are a group of alkaloids; their biogenesis is initiated by the pyridoxal-phosphate catalyzed decarboxylation of L-lysine to cadaverine, which undergoes an enzymatic oxidative deamination to amino aldehyde which cyclizes to the imine form Δ^1-piperideine, followed by a reduction of piperidine which reacts with piperoil-Co-A (piperic acid-coenzyme A ester) forming a piperine [39].

Piperine, accounting for around 98% of the total amount of alkaloids in black pepper, is the most abundant pungent alkaloid; it is responsible for the pungency of pepper. Single crystal X-ray diffraction and mass spectroscopic analysis of yellow prism-like crystal revealed a melting point in the range 128-130 °C. The crystal and molecular structure of piperine (1-piperoylpiperidine) has been discovered after recrystallisation from ethanol oleoresin extract of black pepper, *i.e*. the molecular formula of $C_{17}H_{19}O_3N$ and a molecular mass of 285.1365. It has been revealed that piperine is ternary type of amide which is formed between piperidine in the chair conformation and piperic acid (5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid) [40].

Four geometric isomers of piperine, represented in Fig. (**2**), have been characterized: The major component piperine (*trans-trans* isomer), isopiperine (*cis-trans* isomer), chavicine (*cis-cis* isomer), and isochavicine (*trans-cis* isomer), the last three forms do

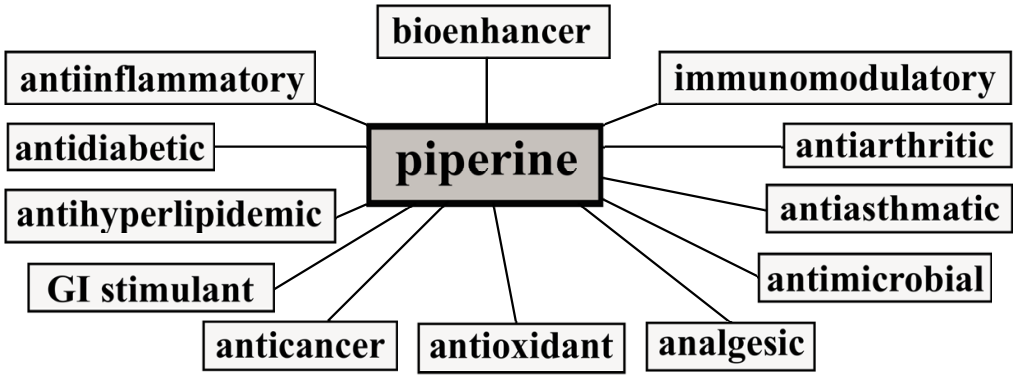

Fig. (1). Potential uses of piperine as nutraceutical.

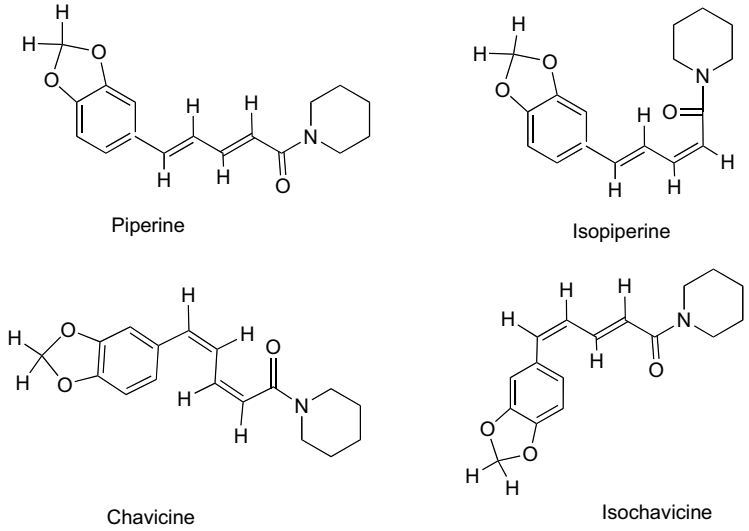

Fig. (2). Geometric isomers of piperine.

not possess any pungent properties [36]. The rate and degree of isomerization are variable. After oral ingestion of piperine in doses of 50 mg, no isomers other than piperine were detected in human plasma (healthy volunteers) [41, 42]. Piperine is a weak base that undergoes hydrolysis and has a limited solubility in water (only 40 mg/L at 18°C) [43]. This is a challenge, but it may be possible to overcome these limitations with nano-scaled formulations for poly-phased delivery systems (*e.g.* solid dispersions, solutions, capsulated vesicles and particles) as well as advanced energy-efficient and environmentally friendly extraction technologies (*e.g.* supercritical fluid extraction, ultrasound and microwave- assisted extractions) [36]. The combination of Accelerated Solvent Extraction (ASE) of piperine and its quantification by thin layer chromatography (TLC) has proved to be an efficient, precise, accurate and cost-friendly analytical technique for the rapid screening of the quality of these kinds of spices [44].

The production of molecular salts of piperine with highly water soluble counter ions, offers an opportunity for increasing the water solubility of piperine *i.e.* these modifications affect its absorption rate and enhance its bioavailability. A recent study on non-covalent interactions between piperine and native β-cyclodextrin underlined the increased water solubility of piperine due to the formation of a piperine-β-cyclodextrin inclusion complex in solution [45].

The native form of the piperidine molecule inhibits UDP-glucose dehydrogenases [46] and specific dehydrogenase complexes associated with the electron transport chain [47]. An assessment of the structure- activity relationship of 38 modified piperine derivatives revealed that the degree of saturation of the unsaturated double bonds in the side chain of the piperidine molecule correlates with enhanced inhibition of rat hepatic constitutive and inducible cytochrome P450 (CYP450) activities. Furthermore, it has been demonstrated that the substituted methylenedioxyphenyl ring and basic piperidine ring contribute to their maximal selectivity [48]. Therefore, these synthetic piperine analogues may be useful in modifying the bioavailability.

Fluorescence titration measurements of two different binding constants of piperine complex with human G-quadruplex DNA sequences correlate to affinity of piperine for two different binding sites. The highest value is a result of the binding of piperine with Pu24T c-myc G-quadruplex DNA as compared to other G-quadruplex DNA *i.e.* differences in sequences and topology lead to reduced affinity in the specific binding in the formation of piperine-DNA complexes [49].

2.2. Absorption and Metabolism of Piperine

The findings regarding the absorption and metabolism of piperine in humans have largely originated from animal studies. In one of the first investigations of piperine metabolism, piperine was administered to male albino rats (170 mg/kg) by gavage and around 97% was found to be absorbed. Approximately 3% of the administered dose of piperine was excreted in the feces, while levels in urine were undetectable, indicating its complete metabolic conversion [50]. Piperine was absorbed very rapidly through the intestinal barrier [51]. After intestinal absorption, due to its insolubility in water, serum albumin is proposed to be the main carrier in its transportation in blood [52]. However, in serum only traces of piperine (< 0.15 %) from 30 min to 24 h after administration were detected, which implies its very low bioavailability and intensive intestinal

metabolism. The main routes of Phase II metabolism of piperine are glucuronidation and sulpha-conjugation. After oral administration of piperine (in rats), the following main metabolites were detected in urine, at 0-96 h after administration: piperonylic acid, piperonyl alcohol, piperonal, and vanillic acid; at 0-6 h after administration, piperic acid could be detected in bile, but no metabolites could be determined in feces [31, 50]. One human study revealed that after intake of piperine capsules (20 mg) for 7 days, piperine did not accumulate in healthy volunteers [53].

2.3. Toxicological Data on Piperine

Piperine can be present in human diet as natural ingredient of black pepper or can be added to food and drink as flavouring agent in different concentrations which can vary extensively, from 0.4-6 ppm (in candies) to 640 ppm (in some baked products) [54]. Regarding the fact that oleoresin of black pepper consists of 40% piperine, individual human exposure to piperine is estimated based on black pepper consumption and is around 1 pg/kg/day [55]. Based on these data, and the fact that piperine is more irritant rather than toxic, its intake from regular food raised no safety concern. No observed adverse effect level (NOAEL) of piperine is 5 mg/kg daily [56].

The pharmaceutical use of piperine is limited due to its low water solubility of only 40 mg/L at 18 °C [43] and putative toxicity (immunotoxicity, reproductive and neurotoxicity) at high concentrations [57, 58]. The different toxicity aspects of piperine have been evaluated in the past years. LD_{50} of piperine in mice and rats is estimated to be 330 and 514 mg/kg, respectively. Doses of 100 mg/kg were found to be nontoxic in subacute toxicity tests [59]. Piperine has been claimed to be a non-genotoxic chemical based on the results from four different types of genotoxicity tests (Ames test, micronucleus test, test for sperm shape abnormality and dominant lethal test) [60-63]. Furthermore, piperine was observed to exert a suppressive effect on the micronucleus formation induced by benzo(*a*)pyrene and cyclophosphamide in mice [64, 65]. In addition, there are studies where the antigenotoxic effects of piperine (provided as a feed additive) against the toxicity of aflatoxin B_1 have been analyzed and it was proposed as being safe with respect to its protective role against aflatoxin-genotoxic action [66].

The immunotoxicity of piperine has been investigated in Swiss male mice where doses of 1.12, 2.25 or 4.5 mg/kg were administered [67]; piperine was assessed to be an immunologically safe compound. Furthermore, piperine was found to possess immunomodulatory properties and to confer protection against the immunotoxicity evoked by different agents, for example, cadmium [68, 69], cypermethrin [70] or deltamethrin [71]. In these studies, piperine has been claimed to be an effective agent in reducing the adverse effects of immunotoxins on apoptosis, blastogenesis, T- and B-cell phenotypes differentiation and cytokine production. Piperine contains a pentacyclic oxindole group in its molecule, which might be related to its possible immunomodulatory properties.

With regard to reproductive toxicity, the majority of studies have been conducted on experimental animals, most usually in rats or Swiss albino mice, *i.e.* a short-term toxicity test was employed for assessing piperine's effects on estrous cycle, spermatogenesis, fertilization, male germ cell, epididymis enzymes, *etc*. Doses up to 75 mg/kg did not induce abnormalities, while at higher doses (100 mg/kg) reductions were observed in the activity of enzymes and sialic acid levels in the epididymis as well as in testis development in pubertal rats [72-74].

3. INTERACTIONS OF PIPERINE WITH OTHER COMPOUNDS

3.1. Piperine-drug Interactions

Beside regular dietary sources, piperine is an ingredient of numerous marketed herbal medicines and food supplements. Excess intake of these products, that provide more than 10 mg of piperine, might result in clinically significant interactions with several drugs [75]. As such, piperine can have a great impact on the bioavailability and pharmacokinetics of other co-administered drugs by modulating their intestinal absorption and metabolism.

There are two possible pathways for nonspecific piperine-drug interactions:

(1) Promoting more efficient absorption of drugs (or some nutrients) by prevention of their non-enzymatic breakdown (decreased secretion of HCl in the stomach) or promotion of active and passive transport mechanisms in the intestine, and

(2) Interaction mechanisms where piperine inhibits drug-metabolising enzymes thus preventing the drug's inactivation and elimination [26, 33, 76].

It has been reported that piperine induces alterations in the villus-crypt structure that alter its fluidity as well as evoking changes in the conformation and activity of enzymes on the outer intestinal membrane. Another possibility to explain piperine's bioenhancing properties is that it changes gastric emptying and transit time to allow increased drug absorption [33, 77-80].

Piperine has been shown to inhibit both *in vivo* and *in vitro* drug-metabolizing enzymes and drug-transporter proteins in a non-specific manner [31, 81-84]. However, it has also been observed to exert stimulating effects on the activity/expression of some enzymes (mainly those in the CYP family) depending on the route, dose and time of piperine exposure [85, 86]. Piperine has been reported to inhibit many important detoxifying enzymes *e.g.* cytochrome P450 family (mainly CYP3A4, also CYP1A1, CYP 1B1/B2, CYP2E1, *etc.*), aryl hydrocarbon hydroxylase (AHH), ethyl morphine-N demethylase, 7-ethoxycoumarin-O-de-ethylase, 3-hydroxy- benzo[*a*]pyrene glucuronidase, uridine diphosphate glucose dehydrogenase (UDP-GDH), uridine diphosphate glucuronyl transferase (UDP-GT), 5-lipoxogenase (5-LOX), cyclooxygenase-I (COX-I). The majority of drugs available today are metabolized by CYP enzymes (*e.g.*, CYP3A4) and UGT, thus studies of possible interactions with piperine have mainly focused on these entities [80, 87, 88].

The inhibitory effects of piperine on the activity and expression of drug-transporter proteins have been investigated mainly with transporters belonging to ATP-binding cassette (ABC) family, like P-glycoprotein (P-gp) [89]. Piperine modulates the activity and expression of P-gp. This interaction is currently a subject of great research interest because of putative alterations of the pharmacokinetic profile and consequent adverse effects of P-gp substrate drugs on one hand, and on the other, the possible beneficial effects in cancer treatment. For example, piperine may enhance the cytotoxicity of anticancer drugs or reverse multidrug resistance in a dose-dependent manner [80]. Beside anticancer agents, there are different classes of drugs, which may interact with piperine: carvedilol, diclofenac, fluvastatin, glimepiride, glipizide, ibuprofen, losartan, naproxen, rosiglitazone, valproic acid; all of them are listed in DrugBank database (www.drugbank.ca/drugs/DB12582).

3.2. Interactions of Piperine with other Nutraceuticals: Focus on Curcumin

Curcumin is a popular and widely investigated nutraceutical, since it exerts antioxidant, anti-inflammatory, antimicrobial and anticancer effects. However, this natural polyphenol has limited therapeutic potential due to its poor bioavailability *i.e.* its limited aqueous solubility, poor absorption, extensive metabolism in liver and intestine, as well as its rapid elimination (for a review see Hewlings and Kalman, 2017, [90]). Piperine has been postulated to overcome the pharmacokinetic drawbacks of curcuminoids like curcumin *e.g.* it was reported to increase its bioavailability up to 2000% in humans [91]. Piperine enhances the absorption of curcuminoids by reducing the activity of glucuronidase both in intestine

and the liver, and by increasing intestinal perfusion [91]. There are a number of convincing reports providing clear evidence that co-administration of curcumin with piperine maximizes its antioxidant, anti-inflammatory effects thus conferring protection against a variety of diseases. In a recent double-blind placebo-controlled study in type 2 diabetes patients, co-supplementation of curcumin with piperine (1000 and 10 mg/day respectively) for 8 weeks led to an enhanced antioxidant capacity as reflected in an elevated serum total antioxidant capacity (TAC), increased activity of superoxide dismutase (SOD) and lowered levels of lipid peroxidation [92]. Consistently, in a randomized double-blind placebo-controlled trial in subjects with the metabolic syndrome (MetS), 8-week supplementation of curcumin (1 g/day) with piperine (10 mg/day) decreased lipid peroxidation and induced SOD activity. Furthermore, in the same study, curcumin and piperine co-supplementation exerted an anti-inflammatory effect as evidenced by a decrease in hs-CRP; it also improved glucose metabolism as revealed by a decrease in HbA1c levels [93]. Increased oxidative stress and inflammation are emerging components of the pathogenesis of exercise-induced muscle damage. The protective role of curcumin and piperine against exercise-induced muscle damage was explored recently in elite rugby players. Daily supplementation with 6 g of curcumin and 60 mg of piperine between 48 h before and 48 h after acute exercise partly attenuated markers of muscle damage and soreness [94]. Among the existing strategies to enhance the bioavailability and antioxidant properties of curcumin, co-administration of curcumin and piperine together with quercetin have been claimed to be potentially beneficial. A combination treatment consisting of curcumin with piperine and quercetin was able to combat impaired SOD and glutathione peroxidase activities, and the reduced glutathione levels evoked by consumption of a high-fat diet or streptozotocin-induced diabetes in rats [95]. Nonetheless, in contrast to these reports, treatment of curcumin with 20 mg/kg piperine to rats with experimentally-induced diabetes did not further improve antidiabetic and antioxidant effects of curcumin; in fact, co-supplementation of piperine at a 40 mg/kg dose abolished the beneficial effects of curcumin [96].

Synergistic interactions of piperine with other nutraceuticals have been reported [26]. In a recent study, four weeks of co-supplementation of resveratrol with piperine potentiated efficiency of low-intensity exercise training on skeletal muscle mitochondrial capacity in healthy young adults [97]. Similarly, compared to single resveratrol supplementation alone, a co-supplementation with piperine increased the bioavailability of resveratrol by 229% and decreased its metabolites approximately 80% [98]. This bioenhancing effect was attributed to inhibiting glucuronidation, and slowing down the elimination of resveratrol by piperine [98]. Moreover, earlier human studies demonstrated that oral supplementation of piperine increased gastrointestinal absorption of beta carotene [99] and elevated plasma levels of co-enzyme Q10 [100].

4. RECENT THERAPEUTIC INSIGHTS OF PIPERINE AS ANTICANCER AGENT: THE IMPORTANCE OF ANTI-OXIDANT POTENTIAL

Oxidative stress, a state of imbalance between the generation of reactive oxygen species (ROS) and the antioxidant defense capacity of the organism, is nowadays known to be involved in many disease processes including cancer, but also atherosclerosis, inflammation, ageing, *etc*. [101-103]. Indeed, high concentrations of ROS are harmful to the organism since they can damage all of the major cellular constituents *e.g*. evoking lipid-, DNA-, protein oxidation resulting in protein misfolding and aggregation [104]. In contrast, at moderate concentrations, ROS play an important role in cellular signaling and are essential in physiological homeostasis. Therefore, increased ROS production during times of increased oxidative stress can disrupt redox regulation of cellular processes [105]. In biological systems, there is a kind of orchestrated synergism, in which a variety of endogenous and dietary antioxidants detoxifies ROS, to combat oxidative stress and maintain redox control [106].

The health benefits of piperine have been attributed to its possible antioxidant properties and inhibitory effects on the production of ROS (see for a review of Srinivasan, 2014) [107]. Earlier studies demonstrated that piperine was a potent inhibitor of oxidative damage capable of preventing lipid peroxidation *in vitro* [108]. Furthermore, piperine inhibited copper-induced lipid peroxidation of human low density lipoprotein (LDL) as reflected in a decrease in the level of thiobarbituric acid reactive substance (TBARS) and relative electrophoretic mobility (REM) of LDL on agarose gel [109]. However, at equimolar concentrations, the inhibitory effect of piperine on oxidation of LDL was less potent than the other compounds studied *i.e*. curcumin, quercetin and capsaicin [109]. Nonetheless, piperine supplementation (0.02 g/kg body weight) for 10 weeks counteracted to a significant extent the increased lipid peroxidation and impaired antioxidant enzyme activities and reduced glutathione (GSH) levels in rats fed a high-fat diet [29]. The protective role of piperine against oxidative stress was confirmed in a recent study, in which piperine at concentrations of 35 and 50 μM improved lipid peroxidation and prevented DNA damage in a cadmium-induced model of oxidative stress in peripheral blood lymphocytes obtained from healthy individuals [110].

The impact of low dose and short term piperine treatment (10 mg/kg/day, intraperitoneally for 14 days) has been investigated in various tissues of rats with streptozotocin-induced diabetes (SID). Treatment with piperine reversed the SID-induced disruptions in oxidative stress and antioxidant defense markers in a tissue-specific manner [111]. Piperine treatment counteracted SID-induced oxidative stress as reflected in the increased oxidized glutathione (GSSG) levels in the brain and the prevention of lipid peroxidation in heart tissue. In addition, piperine supplementation restored the reduced activities of glutathione peroxidase (GSHPx) and SOD in kidney tissue and glutathione reductase in heart tissue. On the other hand, in liver tissue, piperine supplementation did not prevent oxidative stress or reverse the impaired antioxidant defense in SID rats. In fact, it even induced oxidative stress in non-diabetic animals *i.e*. there was an increase in the glutathione oxidation ratio [111]. In another study employing the rat SID model, the effects of different doses of piperine treatment on oxidative stress and antioxidant markers were compared. Lower doses of piperine supplements (20 mg/kg) for 45 days decreased markers of lipid peroxidation and protein oxidation and conversely increased the activities of three antioxidant enzymes SOD, GSHPx and catalase [96]. Notably lower doses of piperine supplementation enhanced GSH, which is an important endogenous antioxidant and a key redox regulator. Surprisingly, at higher piperine supplementation doses (40 mg/kg), these beneficial alterations in oxidative stress markers and antioxidant defense were largely abolished in the SID rats [96]. The same pattern of a dose-dependent dual action of piperine was observed in another study, *i.e*. at lower doses, piperine acted as a hydroxyl radical scavenger, but at higher concentrations, there was evidence of an activated Fenton reaction and increased production of hydroxyl radicals [108]. Nevertheless, a recent study demonstrated that in H661 cells, that have a very low background level of ROS production, piperine treatment between 0.5-100 μM concentrations did not induce any ROS generation [112]. In addition, piperine displayed a moderate H_2O_2 scavenging effect and a minor inhibition of ROS production as revealed by attenuated NADPH oxidase (NOX) activity [112].

These studies provide evidence that the antioxidant properties of piperine may be dose-dependent and tissue-specific. On the other hand, as far as we are aware, there are no human studies performed that uncover the role of sole supplementation of piperine on antioxidant protection against oxidative stress. Nevertheless, several human studies revealed the bioenhancer role of piperine in combination with other antioxidant compounds [97-100]. Therefore,

piperine not only possesses antioxidant effects itself, but it can also potentiate the antioxidant effects of other nutraceuticals as summarized in the previous section.

Piperine's confirmed antioxidative effect could well be linked with chemoprevention. According to current theories, piperine could be effective as an anticancer agent, alone, or in combination with other anti-cancer agents. The mechanism of piperine antitumor effect might be related to its immunomodulatory properties [113], but many reports point out the importance of its antioxidant activity as well as its ability to inhibit the proliferation and survival of various types of cancer cells. Other beneficial properties may be modulation of redox homeostasis, induction of detoxification enzymes, inhibition of angiogenesis, and sensitization of tumors to radiotherapy and chemotherapy [114, 115].

There are many reports investigating the involvement of piperine in combatting carcinogenesis. The effects of piperine on the procarcinogenesis arising from toxicity and genotoxicity induced by aflatoxin B_1 have been investigated for several decades. In an early study, Singh and his collaborators (1994) demonstrated that piperine could reduce the toxicity of aflatoxin B_1, suppressing the toxicity (activation) of the mycotoxin *via* the cytochrome p-450 pathway. This conclusion was achieved using rat hepatoma cells H4IIEC3/G-(H4IIE) and analyzing cellular growth and the formation of micronuclei [116]. Later in 1997, Reen *et al*. reported that piperine was a potent inhibitor of rat CYP4502B1 activity; this prevented the CYP4502B1-mediated activation of aflatoxin B1 in these cells, *i.e.* it acted chemopreventive against the procarcinogenesis mediated through this pathway [117]. Subsequently, Selvendiran and collaborators have examined the chemopreventive/anticancer properties of piperine. They investigated the cytoprotective effect of piperine after inducing an experimental lung cancer in male Swiss albino mice, using benzo[*a*]pyrene, a carcinogen known to cause lipid peroxidation. They monitored the level of lipid peroxidation and the status of several enzymatic antioxidants (SOD, catalase and glutathione peroxidase) and non-enzymatic antioxidants (reduced glutathione, vitamin E, and vitamin C) levels. They reported that piperine, when given by oral route at 100 mg/kg, enhanced the levels of both enzymatic and non-enzymatic antioxidants and suppressed lipid peroxidation not only if the animals were treated with piperine prior to the induction of lung cancer, but also in those receiving piperine after the induction of the lung cancer. Since there was a more pronounced effect in pre-treated animals, it was postulated that piperine exerted chemopreventive effects [118]. In further investigations using the same model, a significant decrease in the levels of lipid peroxidation was observed [119, 120]. These investigators also revealed that levels of protein carbonyls, nucleic acid content and polyamines (*i.e.* factors known to be increased in lung cancer-bearing animals), were lowered after piperine administration. The ability of piperine to inhibit lung metastasis induced by administration of B16F-10 melanoma cells to C57BL/6 mice was studied by Pradeep and Kuttan (2002). They reported that piperine was 100% cytotoxic to B16F-10 melanoma cells at a concentration of 100 μg/mL, while being nontoxic at a tenfold lower concentration. They also observed a significant reduction in lung tumor nodule formation (95.2%) when animals were treated with piperine (200 μmol/kg body weight/dose) simultaneously with the inoculation of the tumor cells in order to generate a metastatic disease. An increase in the life span of these mice was also noted as an outcome of piperine administration. After analyzing several other parameters, such as lung collagen hydroxyproline, lung uronic acid and hexosamine contents, serum sialic acid and serum gamma glutamyl transpeptidase, they concluded that all these metastasis markers significantly declined in response to piperine administration [121].

Bezeera *et al*. (2006), examined the potential of piperine and piplartine to inhibit Sarcoma 180 –initiated tumor growth in Swiss mice, comparing their activity with 5-fluorouracil (5-FU) treatment. A significant reduction in the tumor weight was observed with both administered doses (50 and 100 mg/kg), in piplartine- and piperine-treated animals; there was a tumor growth inhibition ratio of 55.0% and 56.8% for piperine, at doses of 50 and 100 mg/kg, respectively. At a dose of 25 mg/kg per day, 5-FU was observed to reduce tumor weight by 76.7%, over the same time frame [122]. The same group conducted a more extensive analysis of the effects of piperine and piplartine, but in combination with 5-FU. They undertook an *in vitro* test of inhibitory activity in four different tumor cell lines, HL-60, MDA-MB435, SF295 and HCT-8. They observed that the piplartine-5- FU combination exerted more potent *in vitro* cytotoxic effects than piperine-5-FU, although previously both substances were reported to have similar antitumor activities *in vivo* in the Sarcoma 180 model [123]. The antitumor action and immunomodulatory activities of piperine and alcoholic *Piper longum* extracts were examined by Sunila and Kuttan (2004). They reported that piperine alone evoked more potent cytotoxicity to Dalton's lymphoma ascites cells than *Piper longum* alcoholic extract. They also observed an increase in the life span of Ehrlich ascites carcinoma bearing Swiss albino mice. Furthermore, the animals treated with the 1.14 mg intraperitoneal (i.p.) dose of piperine experienced a greater reduction in tumor growth than those receiving the i.p. alcoholic extract of *Piper longum* at a dose of 10 mg [113].

During the current decade, the potential benefits of piperine have been examined in some detail. Lai *et al*. (2012), investigated the effects of piperine on tumor growth and metastasis in 4T1 mammary carcinoma in female BALB/c mice. Piperine was injected into the tumor mass every 3 days on 3 occasions. When injected in two doses, 2.5 and 5 mg/kg piperine showed suppression of primary 4T1 tumor growth in dose-dependent manner. At the same time, administration of 5 mg/kg piperine was reported to significantly inhibit the lung metastasis [124]. In an attempt to reveal the underlying mechanisms of piperine's action, Hwang and his collaborators examined the effects of piperine at preventing tumor invasion and migration using human fibrosarcoma HT-1080 cells. Their study revealed piperine-mediated suppression of the expression of metalloproteinase-9 (MMP-9) at different levels, suggesting that piperine could possibly inhibit tumorigenesis [125]. The antitumor properties of piperine have also been investigated in breast cancer cells by Do *et al*., (2013). They observed that piperine induced growth inhibition and cytotoxicity through both caspase-3 activation and PARP cleavage in several types of human breast cancer cells that were overexpressing HER2. The authors also confirmed the existence of the suppression of EGF-induced MMP-9 expression. In addition, pretreatment with piperine enhanced the sensitization of HER2-overexpressing breast cancer cells to paclitaxel-induced growth inhibition and apoptosis [126].

Further insights into the mechanism of action of piperine have revealed that it can bind to various human G- quadruplex DNA sequences. Tawani and collaborators reported that piperine's anticancer activity could be mediated through the stabilization of the G-quadruplex structure formed at the c-myc promoter region of the DNA sequence (Pu24T), leading to a down-regulation of its expression in cancer cells [49]; this might be the mechanism behind piperine's anticancer activities observed in the above mentioned studies.

5. CURRENT DEVELOPMENTS AND FUTURE PERSPECTIVES

It is of no doubt that the antioxidative effect is the most exploited and most important property of piperine, especially concerning its anticancer activity. However, recent research reveal that also the prooxidant activity of piperine, affecting redox state of cancer cells, might be relevant to its anticancer potential [127]. Namely, the increased oxidative stress results in extreme susceptibility of cancer cells to prooxidant agents that eventually make piperine selectively cytotoxic [115].

A number of investigations have been and still are being performed with a primary goal of defining the biological activities and possible health risks of black pepper and its active principle, piperine. Clinical trials that include sole use of piperine in human subjects are still missing. Nevertheless, evidences from animal studies support its non-toxicity if administered in moderate doses [128, 129].

The main issues that today still limit the wider use of piperine in medical practice, specifically in combination therapy to exploit its bio-enhancer properties, are related to its low bioavailability and potential for drug interactions. These problems still need to be resolved and attention must be paid to the proper dose and dosing regimen, as well as to the design of suitable pharmaceutical formulation(s) that will improve piperine's *in vivo* effects. Currently, the major efforts to transfer piperine from basic research to clinical practice are focused on the development of formulations that improve its low aqueous solubility and poor absorption. For example, one development has been the encapsulation of piperine in lipid carriers (*e.g*., liposomes), or its incorporation in nanoparticles [130-137].

Even though its mechanism of action has not been completely clarified, piperine is established as the first certified bioenhancer due to its ability to reduce the activity of certain enzymes (mainly CYP3A4) and to inhibit P-gp. In this respect, currently its main application in pharmaceutical formulations is as a bioavailability enhancer for different drugs and natural compounds in complex delivery systems. Combinations of piperine and drugs used to treat several major diseases, including tuberculosis, cardiovascular, neurological, and gastrointestinal disorders, are being evaluated (www.drugbank.ca/drugs/DB12582). Since nowadays plant extracts are preferred as active agents, trials are being performed with novel formulations of drug delivery systems loaded with these components [138]. For example, Hoffman *et al*., (2014) have devised pronanolipospheres (PNLs) which consist of orally administrable combinations of lipids, surfactants and co-solvent loaded with piperine [139]. This kind of system would improve the bioavailability of drugs with poor pharmacokinetic properties *i.e*. achieving a reliable absorption of the drug from the GI tract. In the future, other drugs and herbal extracts encumbered by poor intestinal absorption due either to their low permeability or the fact that they are P-glycoprotein substrates may be formulated with piperine *i.e*. piperine can inhibit intestinal P-gp and thus facilitate the drug's absorption. As an example, researchers recently developed a formulation to allow the oral delivery of drugs or herbal extracts with low solubility/permeability after their complexation with piperine in a carbohydrate substrate solubilized with water, ethanol or an organic solvent [140]. This solution was water soluble and not a substrate of P-gp. Moreover, in the most recent studies, piperine has been used as a penetration enhancer, *i.e*. it elevated the flux rate of an active compound (capsaicin) in a transdermal delivery device [141].

These promising results from the nano-formulations of piperine highlight the possibility that new drug delivery systems will become available and piperine's putative health benefits may be fully exploited.

CONCLUSION

Today, there is no doubt that piperine, the most abundant component in species of pepper, possesses optimal characteristics for inclusion into the broad nutraceutical family. Many studies have not only confirmed its antioxidant but also bioavailability enhancer properties for other antioxidants. As research on this topic continue to expand, new opportunities are opening. Progress is made in achieving a deeper understanding of piperine's action, as well as in finding new ways to improve its bioavailability by incorporation into different carrier systems, mostly of a nano-sized nature. In conclusion, the interest in this compound is being redirected, it is no longer simply an interesting component from a well-known pungent spice, and instead, it should be considered as a new and important nutraceutical.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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The Potency of Piperine as Magnesium Bioenhancer in Mice Lung

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Abstract. Magnesium (Mg) deficiency is found in many disease conditions ranging from infectious diseases to degenerative diseases. In COVID-19, magnesium deficiency is associated with susceptibility to infection and the development of severe infections. In this disease, magnesium has been suggested to play a role in the regulation of the immune system, inflammation, and bronchodilatation. Unfortunately, despite its cheap price and abundant availability in nature, magnesium deficiency is difficult to treat. Deficiency persists even after supplementation. Lack of compliance due to side effects at high doses is also another problem. This study aims to investigate the effect of piperine as a magnesium bioenhancer by measuring the bioavailability of magnesium in the lungs of healthy mice using graphite furnace atomic absorption spectrometry (GF-AAS). In addition, two different administrations were compared, ie. Repeated dose oral administrations (5 times/5 days) and single dose intraperitoneal administrations in which each of them consist of 3 testing substances, ie. Magnesium only ($MgSO_4$ 100 mg/kg), piperine only (10 mg/kg), and the combination of magnesium and piperine. One group receives no treatment and serves as a control. The results showed that piperine could increase the uptake of magnesium in the lungs. Surprisingly, this effect is independent of magnesium intake because co-administration of magnesium reduced the uptake of magnesium in the lungs. Of note, single intra peritoneal administration shows a much more prominent effect than repeated doses of oral administration. We suggest piperine could be used as a magnesium bioenhancer in the lung. Future studies using different animal models seem warranted.

Keywords: piperine · magnesium · bioenhancer · in vivo · lung

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1 Introduction

Aging and diabetes are two factors that cause severe attacks in COVID-19 patients. Interestingly, both of these conditions are associated with magnesium (Mg) deficiency. Magnesium is the most abundant intracellular cation after potassium and is involved in $>$ 600 enzymatic reactions in the body, including those that contribute to immune and inflammatory responses in COVID-19 patients [1]. The function of magnesium in the immune response is as a cofactor for the synthesis of immunoglobulins and cofactors of other processes that play a role in the function of T cells and B cells. Regulation of magnesium in immune cells involves several magnesium transport systems [1, 2].

For the treatment of respiratory disease, magnesium sulfate has been widely used in acute severe asthma. As a natural calcium antagonist, the mechanism of action of magnesium in this disease involves inhibition of airway smooth muscle contraction, dilatation of the pulmonary arteries, and reduction of pulmonary artery resistance leading to alleviation of hypoxia [3].

Despite its abundant availability in nature, unfortunately, magnesium deficiency in patients is difficult to treat. This condition occurs because an increase in magnesium intake can also be followed by an increase in its elimination [1]. Therefore, it is necessary to find a way to increase the bioavailability of magnesium, preferably without increasing its side effects, for example, by administering magnesium together with piperine as a bioenhancer.

Piperine is an alkaloid compound that is found in many Piper species plants. These plants have been used for spices and traditional medicine, eg. to treat infections. In the modern world, piperine has been known as a bioenhancer, which is a compound that can increase the bioavailability of several drug compounds and nutrients. There are also commercial preparations of piperine as a bioenhancer (Bioperine®; standardized extract with 95% piperine).

Some of the mechanism of piperine as a bioenhancer involves: 1) efflux-pump inhibition [4]; 2) inhibition of metabolizing enzymes; 3) the thermogenic properties of piperine in the intestine; 4) increase of blood supply; 5) decrease of hydrochloric acid secretion; 6) increase of active and passive nutrient transport; 7) inhibition of drug elimination; and 8) increase of free drug levels by replacing drug binding with plasma proteins [5].

Apart from being a bioenhancer, piperine also has an anti-infective therapeutic effect in vivo at the same dose as a bioenhancer (5–20 mg/kg) [6–9]. Piperine also has activity as an immunomodulator [10]. The nature of piperine as an efflux pump inhibitor indicates the potential of piperine to overcome antibiotic resistance caused by the overactivity of the efflux pump [11].

The benefits of piperine in respiratory disorders have been demonstrated in clinical and pre-clinical trials. In clinical trials, a new formulation of an anti-tuberculosis drug with piperine (Risorine®) has given promising results in the phase III clinical trial [12]. In this clinical trial, piperine 10 mg/kg can reduce rifampicin dose and reduce toxicity during therapy. In pre-clinical trials, piperine inhibited Th-2-mediated cytokine release, eosinophil infiltration, and airway hyper-responsiveness [13].

Although the effectiveness of piperine as a bioenhancer of several organic compounds is known pre-clinically and clinically, the effectiveness of piperine as a bioenhancer of essential minerals, especially magnesium, is still scarce. There is only a report on iron, zinc, and calcium in an ex-vivo study [14]. Similarly, its effect was only reported on iron bioavailability in a clinical study using athletes as the subject [15].

Two ongoing clinical trials are investigating the effects of magnesium and piperine but as separate testing substances. First, they investigate the effects of piperine as a curcumin bioenhancer for the treatment of COVID-19 patients [16], and second, they investigate the combination of magnesium with standard therapy in moderate and severe COVID-19 patients [3].

Taken together, targetting magnesium as a preventive and curative strategy in respiratory disorders seems warranted [17]. Using piperine, this study aims to investigate magnesium bioavailability in lung mice.

2 Material and Methods

2.1 Animals and Treatments

28 male Balb/C mice (The National Agency of Drug and Food Control/ BPOM) were grouped into 7 groups of 4 each, marked with permanent markers, and given food and drink ad libitum in standard mice cages. After acclimatization for 1 week, mice were treated orally (p.o) or intraperitoneally (i.p) as follows:

group without treatment, the piperine group 10 mg/kg i.p, piperine group 10 mg/kg p.o 5 days, group $MgSO_4$ 100 mg/kg i.v, group $MgSO_4$ 100 mg/kg p.o. for 5 days, piperine group dose 10 mg/kg + $MgSO_4$ 100 mg/kg i.v, piperine group dose 10 mg/kg + $MgSO_4$ 100 mg/kg p.o 5 days. Three hours after administration of the last test compound, mice were decapitated with neck dislocation. Lung samples were quickly collected and stored at − 80 ˚C until analysis. The protocol of this study has been approved by LIPI research ethics committee No. 43/klirens/VII/2021.

2.2 Magnesium Analysis

For magnesium analysis with Graphite Furnace Atomic Absorption Spectrophotometer (GF-AAS), lung samples were prepared by wet digestion in concentrated nitric acid (0.2 g samples in 3.6 mL nitric acid) at 120 °C for 30 min using a heat block (ECO COD Thermoreactors) in polypropylene tubes.

2.3 Materials

All materials were obtained from Sigma unless otherwise noted. Piperine was isolated from white pepper (Piper nigrum). $MgSO_4$ (>90%).

2.4 Statistical Analysis

Data are presented as means ± S.E.M of N experiments. GraphPad Prism® Version 5.03 was used for presenting the data and performing statistical analysis. A significance level of 0.05 was chosen for the tests. Analysis of variance (ANOVA) was used for comparing means of more than two groups followed by Tukey's Multiple Comparison Test. The unpaired-T test was used to compare the means between the 2 groups.

3 Results

Our data showed that administration of magnesium alone, both single dose intraperitoneally and multiple doses orally (5×), could not increase magnesium uptake in the lungs (5960 ng/g vs 5616 ng/g vs 5741 ng/g; $p > 0.05$) (Fig. 1a).

Interestingly, a single administration of piperine could increase magnesium uptake in the lungs significantly when given intraperitoneally compared to control (5950 ng/g vs 7672 ng/g; $p < 0.05$). However, this effect was reduced when magnesium was given with piperine concomitantly although a slight increase in magnesium uptake was still observed compared to control (5950 ng/g vs 6254 ng/g; $p > 0.05$) (Fig. 1b.).

Next, we change the dosing regimen to multiple dose oral administration. Similar patterns were observed between multiple oral administration and intraperitoneal administration. Piperine alone showed the highest magnesium uptake (6815 ng/g; $p > 0.05$). However, this effect was almost abolished to a similar value as control when magnesium was given with piperine concomitantly (5950 ng/g vs 5962 ng/g; $p > 0.05$) (Fig. 1c.).

Regarding the dosing regimen of piperine, a single intraperitoneal injection shows a better effect than a repeated dose (5×) of oral administration. However, both regimen is not statistically significant (7672 ng/g vs 6815 ng/g; $p > 0.05$) (Fig. 1d.).

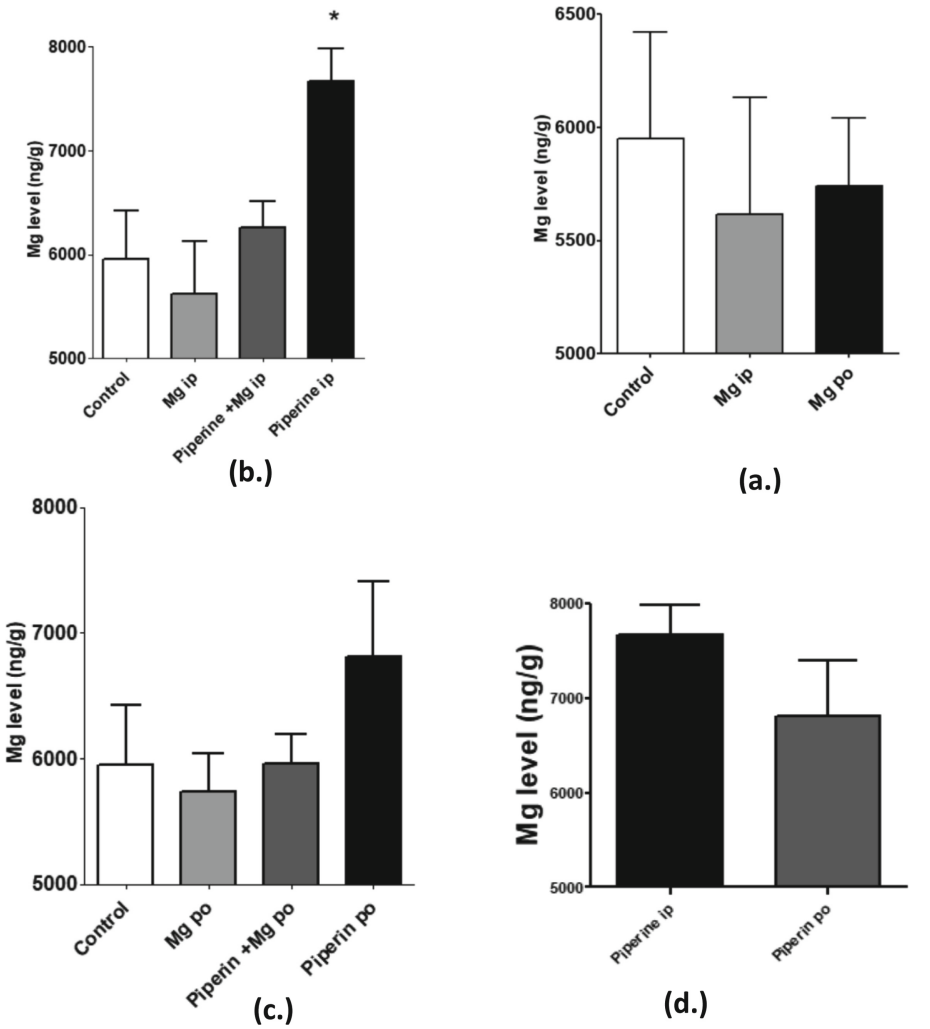

Fig. 1. **a**. Magnesium level in lung sample after oral (po) and ntraperitoneal administration (ip) compared to control. Magnesium administration could not increase magnesium uptake in the lungs. Data were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test ($p > 0.05$ compared to control). **b.** Magnesium level in lung sample. A single dose of piperine could significantly increase magnesium uptake in the lungs when given intraperitoneally. Data were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test (*$p < 0.05$ compared to control). **c.** Magnesium level in lung sample. Multiple doses of piperine slightly increased magnesium uptake in the lungs when given orally. Data were analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test ($p > 0.05$ compared to control). **d.** Magnesium level in lung sample. The different dosing regimen of piperine shows no statistical difference between intraperitoneal and oral administration). Data were analyzed by unpaired t-test ($p > 0.05$).

4 Discussion

Recent studies show that magnesium plays a pivotal role in the severity of COVID-19. This is because some of the risk factors that lead to severe COVID-19, eg diabetes mellitus and cardiovascular disease, were found to be related to magnesium deficiency [18, 19].

There are several ways to alleviate magnesium deficiency, one of them is the use of bioenhancers. One characteristic of bioenhancers is having a sharply strong taste or smell. In this study, we choose piperine. Besides having anti-infective properties, piperine is easily isolated. Therefore, it will be affordable in the future market. Of note, the activity of piperine as magnesium bioenhancer is rarely reported.

Our study showed that magnesium supplementation alone cannot increase magnesium uptake in the lung. This condition is possibly caused by the increase in its clearance. Surprisingly, piperine could increase the uptake of magnesium in the lungs. As predicted, this effect is independent of magnesium intake because co-administration of magnesium reduced the uptake of magnesium in the lungs. This condition is possibly caused by, again, its clearance.

Regarding the dosing regimen, we suggest that the route of administration, instead of total dosing, contributes more to the activity of piperine. The lower activity of piperine as magnesium bioenhancer, when given orally compared to intraperitoneally, is possibly caused by lower piperine blood level. Bhat [20] reported higher liver bioavailability of piperine when given intra peritoneally compared to orally.

Finally, even though oral administration of piperine showed lower magnesium bioenhancer activity, oral administration for future study seems warranted because the differences between oral and intraperitoneal are not statistically different. Of note, oral administration seems more applicable to clinical application.

The limitation of our study is that we use a normal animal. This is however not entirely incorrect because the purpose of this study is to show a clear effect between testing compounds (magnesium alone, with piperine, or piperine alone) and different dosing regimens (single intraperitoneal injection vs multiple doses oral administration). It will be more difficult to study such an effect if we directly use the animal model because so many variables will be involved. In other words, this study serves as a foundation for future studies using animal models.

The use of animal models of diseases is inevitable to study the mechanism of piperine in magnesium regulation because tissue biopsy in the patient is often unethical. In addition, animal models are needed to see the phenotype of this mechanism. Therefore, future studies using different animal models seem warranted.

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Effects of resveratrol alone or in combination with piperine on cerebral blood flow parameters and cognitive performance in human subjects: a randomised, double-blind, placebo-controlled, cross-over investigation

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Abstract

Previous research has shown that resveratrol can increase cerebral blood flow (CBF) in the absence of improved cognitive performance in healthy, young human subjects during the performance of cognitively demanding tasks. This lack of cognitive effects may be due to low bioavailability and, in turn, reduced bioefficacy of resveratrol *in vivo*. Piperine can alter polyphenol pharmacokinetics, but previous studies have not investigated whether this affects the efficacy of the target compound. Therefore, the objective of the present study was to ascertain whether co-supplementation of piperine with resveratrol affects the bioavailability and efficacy of resveratrol with regard to cognition and CBF. The present study utilised a randomised, double-blind, placebo-controlled, within-subjects design, where twenty-three adults were given placebo, *trans*-resveratrol (250 mg) and *trans*-resveratrol with 20 mg piperine on separate days at least a week apart. After a 40 min rest/absorption period, the participants performed a selection of cognitive tasks and CBF was assessed throughout the period, in the frontal cortex, using near-IR spectroscopy. The presence of resveratrol and its conjugates in the plasma was confirmed by liquid chromatography–MS analysis carried out following the administration of 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 with placebo and resveratrol alone. Cognitive function, mood and blood pressure were not affected. The plasma concentrations of resveratrol and its metabolites were not significantly different between the treatments, which indicates that co-supplementation of piperine with resveratrol enhances the bioefficacy of resveratrol with regard to CBF effects, but not cognitive performance, and does this without altering bioavailability.

Key words: **Resveratrol: Piperine: Near-IR spectroscopy: Cognitive performance: Cerebral blood flow**

Resveratrol (3,5,4′-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 human subjects. Of direct relevance here is that these effects include the 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 exerted by resveratrol[3]. These CBF effects are likely to be mediated by the ability of resveratrol to modulate NO synthesis[4], with oral intervention shown to enhance endothelium-dependent relaxation in rats[5,6] and improve flow-mediated dilatation in overweight/obese human subjects[7]. An increase in blood-borne neural metabolic substrates such as oxygen[8] and glucose[9] has been reported to enhance aspects of cognitive performance in healthy, young human subjects. Taken together, it could be hypothesised that an acute increase in CBF, augmenting the delivery of metabolic substrates, might also beneficially affect cognitive performance.

A recent study carried out in our laboratory has demonstrated a dose-related increase in prefrontal cortex CBF during the performance of cognitively demanding tasks in healthy, young adults. This effect was consistent across all time points for 500 mg of resveratrol, but failed to reach significance for 250 mg. The increase in CBF did not facilitate improved cognitive task performance[10]. It was argued that this might be due to the 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 in rodents[11], curcumin in rats

Abbreviations: CBF, cerebral blood flow; NIRS, near-IR spectroscopy; RVIP, rapid visual information processing.

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British Journal of Nutrition

and human subjects[12], and β-carotene following 14 d of co-supplementation in human subjects[13]. Co-supplementation of piperine with resveratrol (10 mg/kg) has been reported to induce 1544 % enhancement of maximum serum resveratrol levels (compared with 100 mg/kg resveratrol alone) and increase exposure (AUC) by 229 % in mice[14]. Potential mechanisms for these phenomena include inhibition of enzymes responsible for the metabolism of polyphenols[14–16], enhancement of metabolism via thermogenic effects[13] and/or competition for membrane efflux pumps in the body and brain: phenomena observed when plant-derived compounds are co-administered, e.g. polyphenols[17]. However, these studies did not investigate whether increased bioavailability leads to increased bioefficacy of the target compound.

Therefore, the present randomised, double-blind, placebo-controlled, cross-over study investigated the effects of 250 mg resveratrol when administered alone and when co-supplemented with 20 mg piperine. The rationale for using 250 mg resveratrol in the present study 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 whether piperine is capable of enhancing the bioefficacy of resveratrol with regard to CBF and cognitive performance in healthy adults. Blood plasma concentrations of resveratrol were measured to investigate whether bioavailability correlated with bioefficacy.

Experimental methods

Participants (cerebral blood flow and cognitive performance assessment)

A total of twenty-three healthy adults (four males and nineteen females, mean age 21 years, range 19–34 years, SD 3·2 years, all right handed) took part in all the three arms of the cross-over study. The data collected from one participant were excluded from the analysis due to data catchment errors. All participants visited the laboratory after a 12 h overnight fast and reported to meet the inclusion criteria, i.e. to be in good health and free from social drug, alcohol, prescription medication, and herbal extract/food supplement use, relevant food allergies, intolerances and digestive problems. A fasted state was considered to be most appropriate due to the individual differences involved in breakfast consumption and the unknowns involved in the absorption of resveratrol together with food. Although food deprivation has been reported to deleteriously affect cognitive function previously in children[18,19], more recent research in athletes during Ramadan has been more ambiguous[20] and a well-controlled study of healthy, young adults has found 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 neurodevelopmental disorder were excluded from participation, as were those who had uncorrected sight problems or were pregnant or seeking to become so.

Participants (bioavailability assessment)

In the bioavailability analysis, six healthy (mean BMI 24·2 kg/m^2, range 21·7–27·2 kg/m^2, SD 2·38 kg/m^2) male adults (mean age 25·8 years, range 23–29 years) took part. Inclusion/exclusion criteria were as per the CBF and cognitive performance aspect of the study.

The present 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 of Northumbria University. Written informed consent was obtained from all subjects. The present trial was registered at ClinicalTrials.gov (study identifier NCT01331382).

Treatments

During the three study visits, the 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, with each combination delivering an inert placebo, 250 mg of *trans*-resveratrol or 250 mg of *trans*-resveratrol plus 20 mg of 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-IR spectroscopy

Relative changes in the absorption of near-infrared light were measured at a time resolution of 10 Hz using a twelve-channel Oxymon system (Artinis Medical Systems B.V.). The system emitted two nominal wavelengths of light (approximately 765 and 855 nm) with an emitter/optode separation distance of 4 cm. The differential pathlength factor was adjusted according to the age of the participant. Relative changes in the concentrations of 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 used (i.e. two channels). The emitter/optode pairs were positioned over the left and right frontal cortices using a standard optode holder headband, which separated the pairs from each other by 4 cm. Therefore, each pair collected data from an area of prefrontal cortex that included the areas corresponding to the International 10–20 system Fp1 and Fp2 electroencephalogram positions. The near-IR spectroscopy (NIRS) data output was time stamped at the start of each task segment to ensure that the data corresponded to the relevant epoch of task performance.

Cognitive tasks

To maximise the cerebral activity-induced modulation of blood flow, a pilot study was initially carried out with a

British Journal of Nutrition

separate cohort of fifteen participants (three males and twelve females, mean age 21·6 years, all right handed) to ascertain the most 'mentally demanding' and 'difficult' tasks from a battery of eleven tasks (data not reported). The five tasks used in the study were all subjectively rated as both the most 'demanding' and most 'difficult' and have all previously been shown to activate the frontal cortex in functional MRI studies[23–25]. A computerised battery of cognitive tasks were delivered using the Computerised Mental Performance Assessment System software.

Serial subtractions. The serial subtraction task consisted of 2 min each of serial 7 s, 13 s and 17 s. The task has been described in detail by Kennedy *et al.* [10].

Rapid visual information processing. The rapid visual information processing (RVIP) task has been described in detail by Kennedy *et al.*[10].

N-back task. The three-back version of the *N*-back task was used in this paradigm, requiring the participants to indicate whether the letter presented on screen was also present three-letter back in the letter sequence. The participants were required to respond by pressing the 'yes' or 'no' button on the response box, to each letter, as quickly as they could. This task includes sufficient stimuli (letters) to last for at least 2 min, 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. The 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 100 mm on-screen line between the descriptors 'not at all' and 'extremely'. They were also required to rate 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 visual analogue scales were scored as a percentage along the line denoting more of the relevant adjective.

Procedure (cerebral blood flow and cognitive performance assessment)

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

On each of the three active study morning sessions, which were conducted 2–14 d apart, the participants visited the laboratory at 08.30 hours in a fasted state and provided confirmation of continued compliance with regard to the inclusion/exclusion criteria. After a 5 min seated resting period, a blood pressure reading was taken, after which the NIRS headband was fitted. The participants then completed a series of mood visual analogue scales and two repetitions of baseline cognitive tasks in the following order: serial 7 s; RVIP; serial 13 s; *N*-back; serial 17 s. The participants then rested for 10 min and a second blood pressure reading was taken. Treatment was then administered, after which the participants sat quietly, watching one of a selection of non-arousing DVD for a 40 min 'absorption' period. Following this, a third blood pressure reading was taken, after which the participants completed four repetitions of the aforementioned tasks in the same order and duration. After the completion of the post-dose tasks, the same mood visual analogue scales were presented and the fourth and final blood pressure readings were taken. NIRS data were captured throughout the

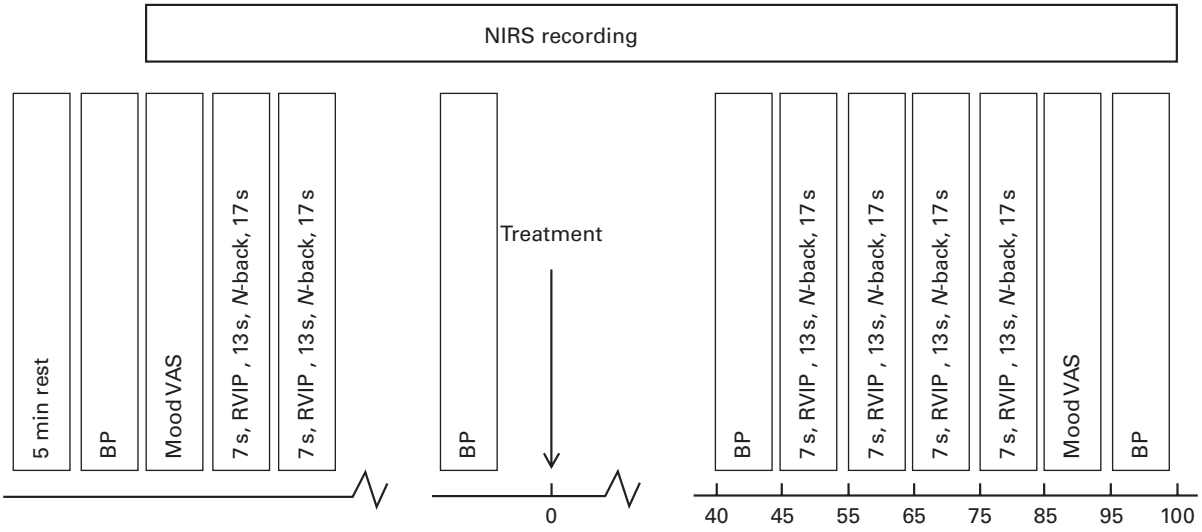

Fig. 1. Timeline and running order of the test sessions. Upon arrival to the laboratory, the participants rested for 5 min before the first blood pressure (BP) reading was taken. The near-IR spectroscopy (NIRS) headband was then fitted. Mood visual analogue scales (VAS) and two repetitions of baseline cognitive tasks were completed, followed by a 10 min rest period. The second blood pressure reading was then taken and treatment was administered. After a 40 min absorption period, the third blood pressure reading was taken. Later, four repetitions of the cognitively demanding tasks were completed, followed by mood VAS ratings and the fourth and final blood pressure readings. RVIP, rapid visual information processing.

sessions. The timeline and running order of the test sessions are shown in Fig. 1.

Procedure (bioavailability assessment)

On each study morning, the participants visited the laboratory at 08.30 hours. Venous blood samples were collected using 4·7 ml monovettes (containing lithium heparin) before the administration of the day's treatment and then 45, 90 and 120 min after the administration of treatment. The samples were centrifuged at 2500 rpm for 15 min at 20°C to obtain plasma, which was then stored at −80°C until analysis.

Preparation of samples

Samples were handled in low-light conditions to reduce the scope for isomerisation. Plasma samples were defrosted at room temperature immediately before extraction, vortexed and then sonicated for 5 min. A 200 μl aliquot was mixed with 900 μl of HPLC-grade ethanol plus 0·1 % formic acid (v/v), along with 100 μl of naringenin internal standard (IS1; Extrasynthese) in ethanol (500 ng/ml). The samples were vortexed, sonicated and then separated via micro-centrifugation at 17 000 ***g*** for 10 min. The supernatant was removed and placed in an amber 1·5 ml centrifuge tube (Eppendorf). The pellet was re-extracted with 1·2 ml of 83 % aqueous ethanol (v/v) following the same protocol. Both extracts were evaporated to dryness under vacuum using a centrifugal evaporator (EZ2+; Genevac) and frozen at −20°C. On the day of analysis, a 70 μl portion of ethanol was added to the secondary extract, which was vortexed and sonicated. A 50 μl aliquot of this solution was then added to the primary extract, which following vortexing and sonication was mixed with 50 μl taxifolin (IS2 at 2 μg/ml; Extrasynthese) in 0·2 % ascorbic acid solution. This solution was vortexed and separated by centrifugation, and the supernatant was placed in an amber vial and analysed via liquid chromatography–MS. Extractions were made in duplicate for each time point. To test the extraction efficiency of this method, blank plasma was spiked with standards at 50 nM, 500 nM, 5 μM and 10 μM concentrations. Across this range, the average extraction efficiencies for *trans*-resveratrol (Cayman Chemicals), resveratrol 3-*O*-sulphate, resveratrol 4′-*O*-glucuronide and resveratrol 3-*O*-glucuronide (Bertin Pharma) were 74, 72, 52 and 55 %, respectively. IS1 and IS2 were extracted consistently at 82 and 100 %, respectively.

Liquid chromatography–MS analysis

Liquid chromatography–MS analysis was conducted using a Shimadzu LC2010CHT HPLC system, consisting of an integrated quaternary pump, a degasser, a chilled autosampler (8°C) and a column oven (30°C), connected to an LCMS2020 single quadrupole mass spectrometer. A 10 μl sample aliquot was separated on an XDB–C18 1·8 μm, 4·6 × 50 mm column (Agilent), running a binary gradient of liquid chromatography–MS-grade water *v.* acetonitrile, both containing 0·1 % formic acid (v/v), running at 0·5 ml/min. The gradient started at 5 % acetonitrile and moved to 10 % at 5 min, 40 % at 20 min and 90 % at 25 min. Following 4 min of washing, the column returned to running 5 % acetonitrile at 30 min and was re-equilibrated over 3 min. The MS analysis was run with an interface temperature set to 350°C, using nebuliser and drying gas flow rates of 1·5 and 15 litres/min, respectively. The analysis was carried out in a negative single-ion monitoring mode, following *m/z* of 403 (glucuronides), 307 (sulphates), 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 was 16 nM for glucuronides, 22 nM for sulphates, and 145 and 290 nM for *cis*- and *trans*-aglycone resveratrol, respectively. Peak areas were normalised to that of IS2 for quantification, while 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 acid and 0·05 % formic acid) to ultraviolet light (254 nm) for 4 h. *Cis*-isomer resveratrol conjugates were quantified as *trans*-isomer equivalents and then summed with the corresponding *trans*-isomers.

Statistical analyses

The analyses of plasma data were carried out with SPSS 16.0 for Windows (SPSS, Inc.) using within-subjects ANOVA (treatment × time) for each metabolite and paired-samples *t* tests to compare AUC, C_{max} and T_{max}, between the two treatments, for each metabolite.

NIRS data were analysed with Minitab 16 for Windows (Minitab, Inc.). For each variable (oxy-Hb, deoxy-Hb and total Hb), data were converted to 'change from baseline' (calculated from a 10 min pre-treatment resting period) and averaged across 2 min epochs during the 40 min 'rest/absorption' and 40 min cognitive task performance periods. The analysis was based on an average of the two NIRS channels to give a measure of cerebral haemodynamics across the prefrontal cortex as a whole, in line with the method of Kennedy *et al.*[10].

The primary analysis of the averaged NIRS data was conducted using within-subjects ANOVA (treatment × 2 min epoch) with *a priori* planned comparisons of data from each epoch being made between placebo and each of the resveratrol treatment groups (250 mg resveratrol and 250 mg resveratrol with 20 mg piperine) using *t* tests calculated with the mean squares error from the ANOVA[26]. To protect against the possibility of type 1 errors, planned comparisons are only reported if they evinced a consistent pattern of significant effects across the analysis period.

Task performance data (also analysed with SPSS 16.0) were analysed as change from pre-dose baseline for each individual task (serial 7 s, RVIP, serial 13 s, 3-back and serial 17 s) using within-subjects ANOVA (treatment × repetition), with planned comparisons for data from each repetition being made as described above.

A power calculation conducted using G* Power[27] suggested that a sample size of twenty-four would be adequate to have greater than an 80 % chance of detecting

the medium effect sizes demonstrated in previous research assessing the effect of resveratrol on NIRS parameters[10].

Results

Near-IR spectroscopy parameters

Total Hb. The ANOVA of total Hb data revealed that there was a significant interaction between the post-dose epoch and treatment ($P<0{\cdot}01$). Planned comparisons revealed that, compared with placebo, treatment with 250 mg resveratrol failed to elicit any modulation of total Hb levels. However, following treatment with 250 mg resveratrol combined with 20 mg piperine, although there were no significant effects during the absorption period, total Hb levels were significantly increased for all task performance epochs (apart from 45, 51 and 79 min). Time points 41, 49 and 61 were all significant at the 0·05 level and the remainder at the 0·01 level.

Oxygenated Hb (oxy-Hb). The ANOVA of oxy-Hb data revealed that there was a significant interaction between the post-dose epoch and treatment ($P<0{\cdot}05$). The pattern was similar to that observed for total Hb, with no modulation being observed following treatment with 250 mg resveratrol, but with significantly increased oxy-Hb levels being observed following treatment with 250 mg resveratrol combined with 20 mg piperine (all epochs, $P<0{\cdot}01$; epochs 45, 49 and 51, $P<0{\cdot}05$; and epoch 79, P=NS).

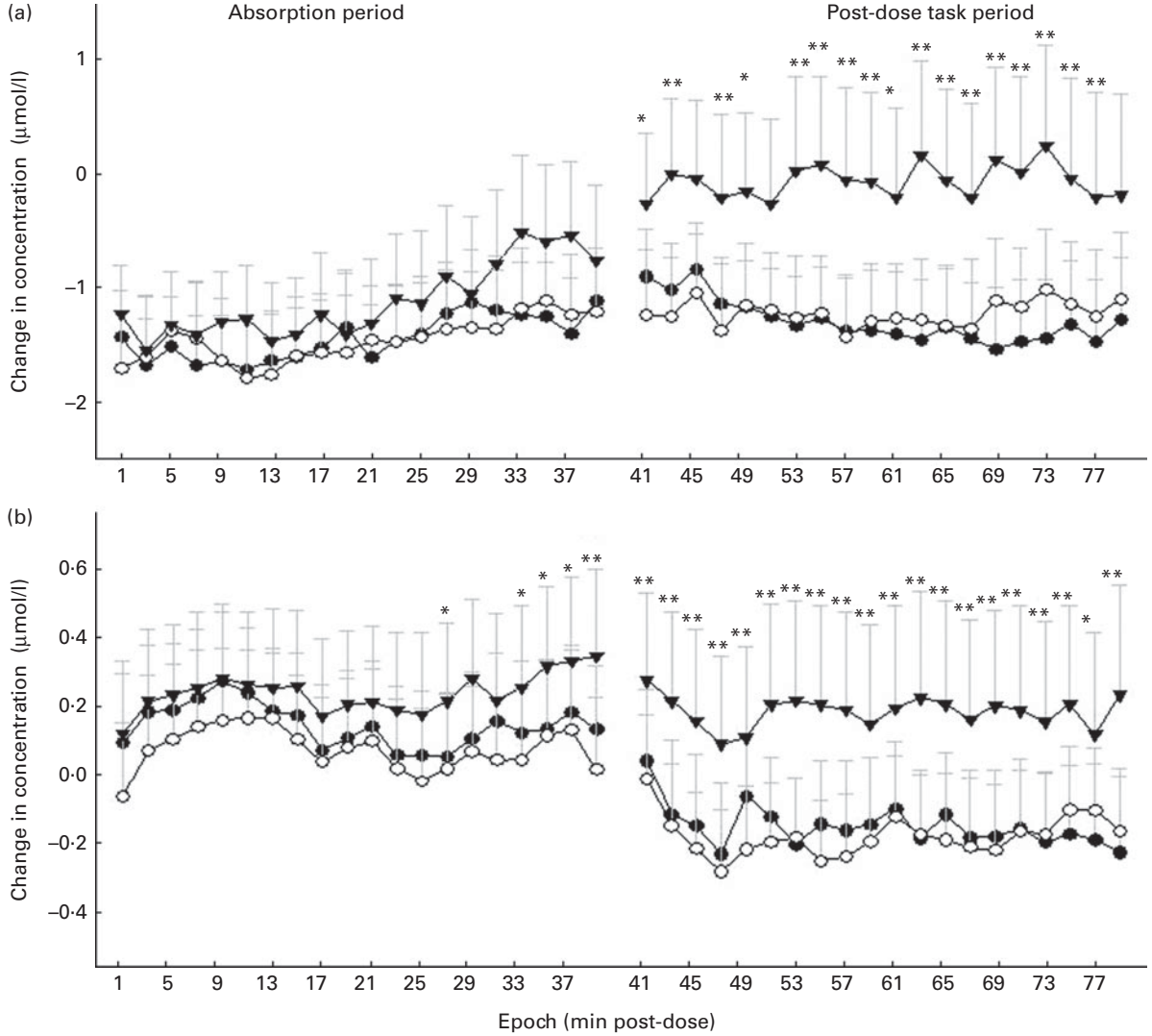

Fig. 2. Haemodynamic effects of 250 mg of *trans*-resveratrol alone and when co-supplemented with 20 mg of piperine in healthy, young human subjects. Changes in the concentrations of (a) total Hb and (b) deoxygenated Hb (deoxy-Hb) during a 40 min absorption period and subsequent 40 min of cognitive task performance following the administration of placebo (○), 250 mg *trans*-resveratrol (●) and 250 mg *trans*-resveratrol with 20 mg piperine (▼). The study followed a cross-over design (n 23 per condition). Data were averaged across 2 min epochs. *A priori* planned comparisons between data from each resveratrol group and those from the placebo group for each epoch were made using t tests by incorporating mean squares error from an initial ANOVA. Values are means, with their standard errors represented by vertical bars. Mean value was significantly different from that of the placebo group: * $P<0{\cdot}05$, ** $P<0{\cdot}01$.

British Journal of Nutrition

Table 1. Effects of resveratrol on cognitive performance

(Mean values with their standard errors; *n* 23)

		Task battery repetition												
		Baseline		1		2		3		4		ANOVA		
Measures	Treatment condition	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Effect	*F*	*P*
7 s Correct (*n*)	250 mg resveratrol	28·85	2·75	1·20	1·02	1·98	0·94	1·54	0·83	0·80	1·16	T	0·252	0·778
	250 mg resveratrol with 20 mg piperine	28·83	2·59	1·52	0·85	−0·04	0·94	0·39	1·25	0·57	1·13	R	0·487	0·692
	Placebo	28·85	2·04	1·94	1·12	0·89	1·11	0·11	1·43	0·98	1·29	T × R	0·675	0·606
7 s Incorrect (*n*)	250 mg resveratrol	1·87	0·30	0·35	0·51	−0·26	0·37	0·35	0·38	0·70	0·55	T	0·517	0·600
	250 mg resveratrol with 20 mg piperine	1·67	0·23	0·11	0·39	0·67	0·38	1·33	0·52	1·07	0·49	R	2·09	0·110
	Placebo	1·91	0·26	0·30	0·52	0·30	0·47	0·78	0·60	0·13	0·46	T × R	1·02	0·416
13 s Correct (*n*)	250 mg resveratrol	22·22	2·25	0·70	0·88	−0·78	0·90	0·22	0·87	−1·17	0·98	T	1·68	0·199
	250 mg resveratrol with 20 mg piperine	22·46	2·17	1·33	0·75	−1·15	1·26	−0·11	1·25	1·07	0·87	R	3·17	0·030*
	Placebo	21·83	1·60	3·26	0·83	0·78	1·41	1·17	1·20	1·09	1·03	T × R	0·644	0·695
13 s Incorrect (*n*)	250 mg resveratrol	2·04	0·23	0·13	0·40	2·04	1·00	0·65	0·51	1·17	0·47	T	0·969	0·388
	250 mg resveratrol with 20 mg piperine	1·89	0·36	0·11	0·44	1·59	0·94	1·59	0·63	0·76	0·73	R	7·08	<0·001**
	Placebo	2·39	0·36	−1·09	0·33	0·96	0·84	0·78	0·53	0·44	0·58	T × R	0·445	0·765
17 s Correct (*n*)	250 mg resveratrol	17·22	1·68	1·39	0·71	1·48	0·81	2·35	0·75	1·09	1·13	T	0·405	0·670
	250 mg resveratrol with 20 mg piperine	17·78	1·61	0·39	0·63	0·44	0·86	0·87	0·90	2·13	0·76	R	0·502	0·638
	Placebo	16·80	1·29	1·72	0·62	1·37	0·68	1·15	0·95	1·89	0·59	T × R	1·07	0·383
17 s Incorrect (*n*)	250 mg resveratrol	2·28	0·28	0·15	0·41	0·02	0·47	0·24	0·52	1·54	1·09	T	0·719	0·493
	250 mg resveratrol with 20 mg piperine	2·17	0·29	0·30	0·42	0·30	0·50	0·57	0·42	0·52	0·37	R	1·41	0·254
	Placebo	2·57	0·27	−0·30	0·37	−0·44	0·45	0·44	0·67	−0·04	0·36	T × R	0·791	0·578
N-back accuracy (%)	250 mg resveratrol	93·38	1·17	−0·34	0·97	−1·02	1·05	−0·92	1·08	−0·05	1·00	T	0·617	0·544
	250 mg resveratrol with 20 mg piperine	94·40	0·91	−2·03	1·02	−1·84	1·09	−0·29	0·89	−1·45	1·27	R	0·274	0·844
	Placebo	94·40	0·74	−1·26	1·08	−1·55	0·92	−2·61	1·13	01·45	0·93	T × R	0·678	0·599
N-back reaction time (ms)	250 mg resveratrol	1540·45	145·80	−291·04	48·75	−345·87	53·98	−312·95	52·58	−398·24	58·12	T	1·28	0·288
	250 mg resveratrol with 20 mg piperine	1476·26	189·03	−243·72	67·01	−287·30	77·69	−375·74	94·44	−292·16	70·96	R	3·93	0·012*
	Placebo	1475·04	161·35	−194·12	34·69	−149·39	70·65	−264·79	81·89	−271·44	57·14	T × R	1·12	0·347
RVIP correct (%)	250 mg resveratrol	71·06	3·76	0·41	2·98	−4·48	2·44	−7·47	3·73	−7·76	2·58	T	1·17	0·321
	250 mg resveratrol with 20 mg piperine	65·81	4·00	3·76	2·32	1·31	3·39	−4·36	3·39	−1·68	3·51	R	7·58	<0·001**
	Placebo	69·16	3·90	1·50	2·25	−7·38	3·65	−7·47	2·51	−6·66	3·40	T × R	0·489	0·816

T, treatment; R, repetition; RVIP, rapid visual information processing.
There was a significant main effect for R: * $P<0·05$, ** $P<0·01$.

Deoxygenated Hb (deoxy-Hb). The ANOVA of deoxy-Hb data revealed that there was no significant main effect or interaction between time and treatment. 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 treatment with 250 mg resveratrol combined with 20 mg piperine, deoxy-Hb levels were significantly increased in comparison with those observed after placebo administration (absorption period: epochs 27, 29, 33, 35 and 37, $P<0{\cdot}05$ and epoch 39, $P<0{\cdot}01$; post-dose task period: all epochs, $P<0{\cdot}01$ and epoch 77, $P<0{\cdot}05$).

The mean data (with their standard errors) and the results of the planned comparisons for total Hb and deoxy-Hb are shown in Fig. 2.

Cognitive task performance and mood

There were no significant treatment-related differences in any cognitive or mood measures. The raw baseline task scores and mood ratings and changes from baseline mean task scores and mood ratings are given in Tables 1 and 2, respectively.

Blood pressure

No significant treatment-related differences were observed in pulse rate and diastolic or systolic blood pressure. The raw baseline blood pressure readings and changes from baseline post-dose blood pressure readings are given in Table 3.

Bioavailability

No resveratrol (in any form) was found in baseline samples, indicating that none of the participants consumed resveratrol before the start of the study. Following oral intervention with 250 mg of resveratrol, plasma concentrations of total resveratrol metabolites ranged from 2 to 18·2 μM, varying between the participants and treatments. However, no aglycone *trans*- or *cis*-resveratrol was quantifiable in plasma. Resveratrol 3-*O*-sulphate was the predominant metabolite in all participants, contributing 59–81 % of total metabolites. The 4′-*O*-glucuronide and 3-*O*-glucuronide forms made roughly equal contributions to the remaining metabolites in circulation. C_{max} was typically achieved at 90 min. Resveratrol conjugates were present in plasma as both *trans*-isomers and *cis*-isomers, varying between the participants. The average C_{max} *trans:cis* ratios for resveratrol 3-*O*-sulphate and resveratrol 3-*O*-glucuronide following the consumption of all *trans*-resveratrol were 4·7 (SEM 5·6) (range 1·2–15·9) and 5·1 (SEM 5·6) (range 0·94–18·8), respectively. *Cis*-resveratrol 4′-*O*-glucuronide was found in some, but not in all subjects. Extraction efficiency tests did not indicate significant induction of isomerisation during sample handling, suggesting that this conversion occurs *in vivo*.

Although the average concentrations of resveratrol 3-*O*-sulphate, 4′-*O*-glucuronide and 3-*O*-glucuronide at C_{max} appeared to be lower following the co-supplementation of resveratrol with piperine compared with those following supplementation of resveratrol alone, there was no significant difference between the treatments. Similarly, there was no significant

Table 2. Effects of 250 mg resveratrol alone and when co-supplemented with 20 mg piperine on mood in healthy, young human subjects (Mean values with their standard errors; *n* 23)

		Baseline		Post-dose		ANOVA		
Measures	Treatment condition	Mean	SEM	Mean	SEM	Effect	*F*	*P*
Alert	250 mg resveratrol	50·83	3·79	−6·65	5·44	T	0·767	0·470
	250 mg resveratrol with 20 mg piperine	49·13	3·78	4·43	4·07	R	0·359	0·555
	Placebo	51·57	4·08	−4·87	4·68	T × R	3·28	0·047*
Jittery	250 mg resveratrol	16·83	2·91	19·78	5·40	T	0·532	0·591
	250 mg resveratrol with 20 mg piperine	18·61	3·33	20·48	4·95	R	25·79	<0·001**
	Placebo	15·39	2·54	20·87	4·73	T × R	0·022	0·979
Mental fatigue	250 mg resveratrol	28·96	4·69	35·65	6·18	T	0·839	0·439
	250 mg resveratrol with 20 mg piperine	27·48	4·86	32·48	5·93	R	45·47	<0·001**
	Placebo	26·22	4·10	33·74	6·11	T × R	0·147	0·864
Overall mood	250 mg resveratrol	62·87	3·46	−16·13	4·48	T	2·66	0·081 t
	250 mg resveratrol with 20 mg piperine	64·48	3·04	−12·78	3·60	R	25·87	<0·001**
	Placebo	67·35	2·71	−13·74	2·97	T × R	0·321	0·727
Relaxed	250 mg resveratrol	62·91	2·67	−24·52	5·62	T	0·566	0·572
	250 mg resveratrol with 20 mg piperine	60·35	3·29	−14·13	6·00	R	20·70	<0·001**
	Placebo	62·52	1·98	−20·61	4·44	T × R	1·79	0·179
Tense	250 mg resveratrol	25·48	3·29	25·74	6·35	T	2·32	0·110
	250 mg resveratrol with 20 mg piperine	23·87	3·28	26·35	6·40	R	26·08	<0·001**
	Placebo	19·83	3·02	25·30	5·37	T × R	0·016	0·984
Tired	250 mg resveratrol	47·09	4·51	14·57	5·33	T	0·405	0·669
	250 mg resveratrol with 20 mg piperine	50·74	5·05	4·04	3·92	R	5·96	0·023*
	Placebo	45·57	4·42	11·52	6·39	T × R	1·72	0·191

T, treatment; R, repetition; t, trend.
There were significant main effects for R and the T × R interaction: * $P<0{\cdot}05$, ** $P<0{\cdot}01$.

British Journal of Nutrition

British Journal of Nutrition

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

		Task battery repetition						ANOVA		
		Baseline†		PD 1†		PD 2†				
Measures	Treatment condition	Mean	SEM	Mean	SEM	Mean	SEM	Effect	*F*	*P*
Systolic blood pressure (mmHg)	250 mg resveratrol	112	1·98	2·35	1·77	4·87	1·21	Tr	0·621	0·542
	250 mg resveratrol with 20 mg piperine	114·17	1·98	1·39	1·26	4·90	1·72	Ti	9·61	0·005**
	Placebo	113·22	2·31	−0·04	1·78	3·39	2·13	Tr × Ti	0·089	0·915
Diastolic blood pressure (mmHg)	250 mg resveratrol	75·65	1·66	2·57	0·90	4·17	0·96	Tr	3·68	0·045*
	250 mg resveratrol with 20 mg piperine	75·09	1·62	4·83	1·38	4·70	1·65	Ti	0·628	0·437
	Placebo	76·91	2·48	−0·17	2·08	0·65	1·77	Tr × Ti	0·258	0·724
Pulse rate (bpm)	250 mg resveratrol	68·43	2·48	−0·83	1·07	−2·26	1·51	Tr	1·77	0·192
	250 mg resveratrol with 20 mg piperine	67·91	2·14	0·35	1·87	−3·74	3·78	Ti	3·38	0·080 t
	Placebo	70·87	2·29	−3·78	1·63	−6·87	1·63	Tr × Ti	0·368	0·584

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, 40 min post-dose and immediately before post-dose tasks; PD 2, 95 min post-dose and immediately after post-dose tasks.

difference in the values of area under the curve, and there was no significant change in T_{max} between the treatments.

The mean plasma concentrations of *trans*-resveratrol 3-*O*-sulphate and combined 4′-*O*-glucuronide and 3-*O*-glucuronide metabolites at pre-treatment and at 45, 90 and 120 min post-dose time points, for both treatments, are shown in Fig. 3.

Discussion

The present study demonstrates that the well-established bioenhancer piperine can increase the bioefficacy of the polyphenol resveratrol when co-supplemented in healthy human subjects. Whereas 250 mg of orally administered *trans*-resveratrol had no significant effects on overall CBF (total Hb) during the performance of cognitively demanding tasks, co-administration of the same dose of resveratrol with 20 mg piperine resulted in significantly increased CBF for the duration of the 40 min post-dose task period. The findings with regard to the supplementation of resveratrol alone in this respect are broadly in line with the dose–response pattern of CBF observed following resveratrol administration in a previous study, in which a dose of 250 mg was largely ineffective[10]. Despite this piperine-mediated enhancement of the CBF effects of resveratrol, there were no significant treatment-related differences in the performance of the cognitive tasks, blood pressure/heart rate or participants' ratings of mood for either active treatment.

The pattern of haemodynamic effects of resveratrol observed in the present study, when supplemented with piperine, is exactly in line with that in the aforementioned previous resveratrol intervention study following the administration of a 500 mg dose[10]. This pattern is observed as significantly higher levels of total Hb and oxy-Hb, alongside deoxy-Hb, during the post-dose cognitive task period and represents increased CBF and oxygen utilisation, respectively. This haemodynamic response is dissimilar to that observed during cognitive task performance alone. Here, total Hb and oxy-Hb levels typically rise alongside a concomitant decline in deoxy-Hb levels[28], with this phenomenon being predicated based on the fact that neural activation instigates an increase in CBF that is greater than the metabolic rate of oxygen extraction/utilisation. As such, deoxy-Hb levels can be observed to decrease during cognitive performance[29]. The different deoxy-Hb response observed following resveratrol treatment is probably predicated based on indirect effects on mitochondrial phosphorylation. In support of this, Lagouge *et al.*[30] reported that in mice supplemented with 400 mg/kg/d resveratrol, for 15 weeks, significantly increased mitochondrial structures and enzymatic activity. This resulted in a significant increase in VO_2 and $VO_{2\,max}$ rates and was observed to increase running time and tolerance to cold. In terms of mechanisms, resveratrol can interact with the sirtuin ('silent information regulator'; SIRT) system, a class of proteins involved in multifarious biological processes that has received a great amount of attention over the past decade in relation to life extension[31]. Of importance here is that SIRT is implicated in the deacetylation of PPARγ co-activator 1-α (*PGC-1α*), a gene that controls mitochondrial biogenesis and function[32],

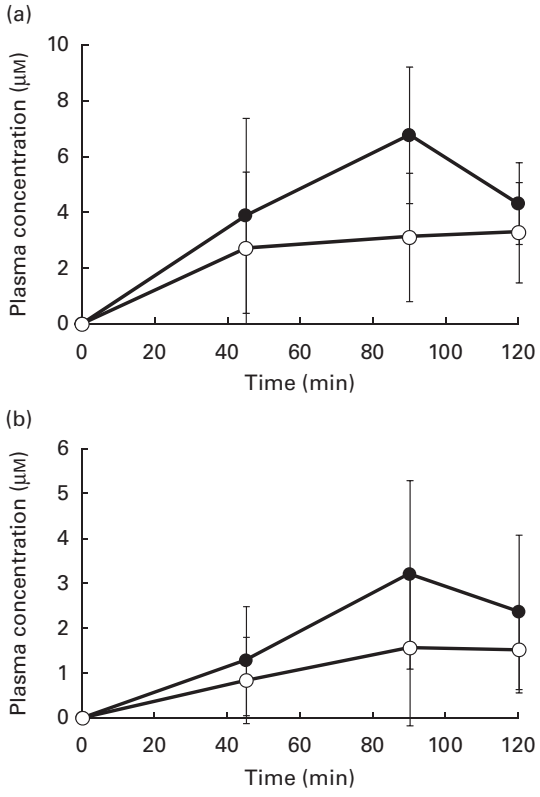

Fig. 3. Plasma bioavailability of resveratrol metabolites following (a) the administration of 250 mg *trans*-resveratrol alone and (b) the administration of 250 mg *trans*-resveratrol with 20 mg piperine in healthy, young human subjects. Values are means (*n* 6), with their standard errors represented by vertical bars. ●, Concentration of resveratrol 3-*O*-sulphate; ○, combined concentrations of resveratrol 4′-*O*-glucuronide and resveratrol 3-*O*-glucuronide.

and while the oxygenation effects in the above-mentioned study in rodents were observed following chronic consumption, these mechanisms would explain the VO_2 effects observed in the present study, represented by deoxy-Hb.

Interestingly, in light of the significant CBF effects occurring only with the resveratrol/piperine combination, no significant differences were observed in the plasma concentrations of resveratrol between the treatments. In both treatment conditions, resveratrol metabolites were present in the plasma across the post-dose cognitive task period and the parent compound was unquantifiable at all time points. However, contrary to the hypothesis of piperine-induced bioenhancement, the pattern of effects observed in the present study actually suggests inhibition rather than enhancement of plasma concentrations; for example, the C_{max} of total metabolites after treatment with 250 mg resveratrol was 9·98 μM compared with 4·82 μM in the piperine co-supplemented condition. Piperine also appeared to be inhibiting the transit of resveratrol, evidenced by the T_{max} of metabolites in the 250 mg resveratrol condition occurring at the 90 min sample time point compared with the 120 min time point in the co-supplemented condition and the observation of metabolite concentrations reducing at the 120 min time point in the 250 mg resveratrol condition and not in the co-supplemented condition. Nevertheless, this pattern of effects exhibited no significant differences between the treatment groups, which suggests two possibilities: either piperine can exert CBF effects independently of resveratrol or, alternatively, it potentiates the effects of resveratrol observed previously on CBF.

Taking the first of these possibilities into account, it is notable that there is only one study[33] that suggests that piperine is capable of interacting with NO and that this is the inducible NO synthase isoform that 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 the present study, and this precluded the need for a piperine-only treatment condition in the study. The exception here is a small amount of literature in rats that 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 to enhanced cognition *per se*[35–37]. Nevertheless, future studies investigating the efficacy of piperine alone on these parameters, in human subjects, to clarify this issue are warranted.

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 being observed in the resveratrol condition alone (a finding mirrored in the study of Kennedy *et al.*[10] with the same dose), it seems more likely that piperine increases 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 (500 mg), can increase CBF in healthy human subjects[10].

Of the potential mechanisms to explain the efficacy-enhancing effects of piperine, one is that piperine is able to enhance the activity of resveratrol, the neuronal vasculature, and/or some other factor relevant to CBF through its thermogenic properties. As evidence of the heat-proffering properties of piperine, specifically in neural tissue, Reanmongkol *et al.*[38] reported on the ability of piperine to stimulate the 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 β-carotene concentrations in human subjects[13] via an increase in the absorption rate of the intestinal epithelium and, as a mechanism, could exist without piperine inducing an overall increase in the bioavailability of resveratrol: a phenomenon observed previously[11–14], but not replicated in the present study.

In terms of behavioural effects, the results of the present study are in line with previous findings, i.e. a lack of any effect of a 250 mg dose of resveratrol with regard to cognitive task performance[10]. One of the primary reasons for using piperine in the present study was to ascertain whether this well-established bioenhancer of polyphenols also induces the enhancement of resveratrol's bioefficacy, especially in terms of cognitive function due to the null effects reported previously. However, while the increase in CBF during task performance was potentiated by piperine, the pattern was largely the same as that observed following the administration of

British Journal of Nutrition

a larger dose of resveratrol (500 mg in Kennedy *et al.*[10]), where cognitive effects were also lacking. Therefore, it would appear that acute increases in CBF are not sufficient, in themselves, to alter cognitive function in the young, healthy cohorts examined in the present study 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 exhibiting age- or pathology-related decrements in CBF and cognitive function.

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

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114514000737

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None of the authors has any conflicts of interest to declare.

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British Journal of Nutrition

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