

Potential Physiological Importance of Pyrroloquinoline Quinone

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Abstract

Pyrroloquinoline quinone (PQQ) is a novel biofactor for which a proposition can be made for physiological importance. PQQ was first recognized as an enzyme cofactor in bacteria. It has recently been tentatively identified as a component of interstellar dust. Thus, PQQ may have been present throughout early biological conception and evolution. PQQ is also a potent plant growth factor. Consequently, for animals and humans, there has been constant exposure to PQQ. In animals, PQQ is reported to participate in a range of biological functions with apparent survival benefits (e.g., improved neonatal growth and reproductive performance). There are also benefits from PQQ supplementation related to cognitive, immune, and antioxidant functions, as well as protection from cardiac and neurological ischemic events. Although PQQ is not currently viewed as a vitamin, its involvement in cell signaling pathways, particularly those important to mitochondriogenesis in experimental animal models, may eventually provide a rationale for defining PQQ as vital to life. For humans, such evidence suggests there may be similar parallels or benefits from improving PQQ status. (*Altern Med Rev* 2009;14(3):268-277)

Introduction

Pyrroloquinoline quinone (PQQ) was first recognized as a bacterial cofactor by Hauge,¹ and later by Anthony,²⁻⁴ Salisbury,^{5,6} Duine,⁷ and their co-workers. PQQ, also known as methoxatin (Figure 1), is water soluble and heat stable. Under appropriate conditions, PQQ is capable of catalyzing continuous redox cycling (the ability to catalyze repeated oxidation and reduction reactions), as well as oxidative deaminations.⁸

These chemical properties are novel in many respects. For example, in chemical assays, PQQ's stability renders it capable of carrying out thousands of redox catalytic cycles; whereas, other bioactive quinones capable of redox cycling (e.g., epicatechin) tend to self oxidize and/or form polymers (e.g., tannins). Table 1 contains data that in part demonstrates the effectiveness of PQQ as a redox cycling agent.⁸⁻¹³ There is also a range of papers that describe PQQ's use in an analytical setting.¹⁴⁻¹⁷ PQQ molecules can be immobilized and fixed at the surface of analytical electrodes. When coupled to appropriate enzyme systems, highly specific and sensitive assays have been developed to assay compounds ranging from glucose to common narcotics.¹⁴⁻¹⁷ As will be highlighted in subsequent sections, the novel chemical attributes of PQQ help explain many of its metabolic and health-related features.

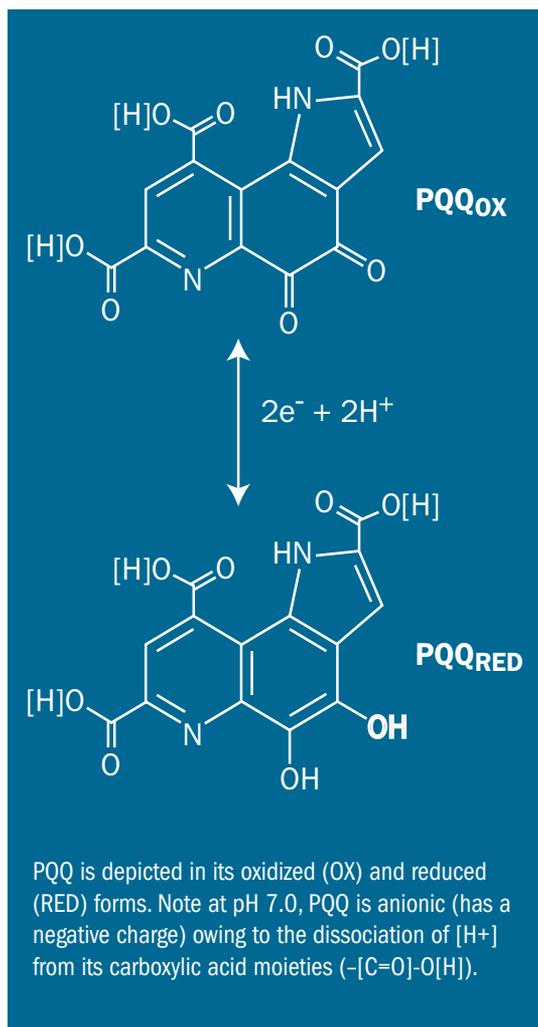
Biochemical Roles and Mechanisms Evolution and Its Functions in Bacteria and Plants

To make the case for physiological and biomedical importance in humans, it is important to note that many of PQQ's functions are universal. For example, for many bacterial species, PQQ stimulates growth and serves as a cofactor for a special class of dehydrogenases/

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Figure 1. Structure of PQQ

oxidoreductases. Enzymes containing PQQ are sometimes designated quinoproteins.⁸ Although the “quinoproteins” include many types of quinone-containing proteins and enzymes, the PQQ-requiring glucose and alcohol dehydrogenases are distinguished, because the PQQ associated with these proteins is dissociable and synthesized in metabolic pathways that can be separately controlled from those pathways important for the generation of the eventual targeted protein.¹⁸⁻²⁰ In addition to a cofactor role, PQQ can also be thought of as a trophic factor important to the growth and metabolism of bacteria, particularly methylotrophic bacteria (bacteria capable of growing on simple carbon sources).

From an evolutionary perspective, current evidence suggests PQQ is a component of interstellar dust as analyzed by particle impact time-of-flight mass spectrometry.^{21,22} Cometary grains are considered to be the precursors of organic materials in early life on the earth. It can also be argued that strong redox catalysts would be required to trigger the earliest chemical evolutionary steps. The presence of PQQ in stellar dust raises the question of PQQ's evolutionary importance to simpler life forms, given its wide range of chemical properties, such as redox catalysis and the ability to carry out useful amino acid modifications (e.g., oxidative deamination reactions).

At the next level it is important to highlight the symbiotic relationship between plants and soil bacteria, such as rhizobacterium.²³⁻²⁵ Plants cultivated in hydroponic culture systems with rhizobacterium have significantly increased height, flower number, fruit number, and total fruit weight; whereas, this does not occur with genetically modified rhizobacteria unable to produce PQQ.²³ PQQ added directly to hydroponic culture systems also confers a significant increase in the fresh weight of seedling plants. In part, the role of PQQ is related to phosphate uptake by plants, because PQQ, as a cofactor for rhizobacteria dehydrogenases, facilitates making soil and the local environment more acidic.²⁴ As a consequence, phosphate is made more available to plant roots. In addition, independent roles have been proposed for PQQ related to plant growth via activation of cell signaling, antioxidant defense, and viral protection.²⁵

For humans and animals, the ubiquitous presence of PQQ in common types of bacteria, soil, and plants suggests constant exposure to PQQ. PQQ has been found in all plant foods analyzed to date.^{8,26-28} In this regard, it is interesting to note that although many bacteria make PQQ, this is not the case for common intestinal bacteria, such as *Escherichia coli*. *Escherichia coli* can synthesize PQQ-dependent enzymes capable of utilizing PQQ under certain nutrient limiting conditions;²⁹ however, the enzymes only become functional when PQQ is present. Hence, an external source of PQQ may be important in sustaining human and animal tissue levels of PQQ, as well as maintaining an optimal enteric environment.

Table 1. PQQ as a Redox Cycling Agent

Compound	Potential Number of Catalytic Cycles
PQQ	20,000
Quercetin	800
Catechin	75
Epicatechin	700
Norepinephrine	200
Epinephrine	100
DOPA	20
6-OH-DOPA	20
Ascorbic Acid	4

Redox cycling systems result in repeated chemical reactions in which molecules that act as catalysts are repeatedly oxidized and/or reduced. The potential number of catalytic cycles (number of repeated reactions) depends in part on chemical stability. PQQ is relatively stable; whereas, self-oxidation, polymerization, and/or changes in chemical structure are factors that compromise the chemical stability of many bioactive quinones or enediols (e.g., ascorbic acid). Details of the redox cycling system and basis for defining the relative number of catalytic cycles may be found in references 8, 13 and 67.

Mechanisms and Proposed Functions in Humans and Animals

A number of physiological properties have been attributed to PQQ, ranging from classical water-soluble vitamin/cofactor functions to those important to antioxidant potential.^{8,30-39} While a role as a vitamin in animal or human nutrition seems unlikely at this time, similar to other polyphenolic biofactors, there is strong evidence PQQ may play an important role in pathways important to cell signaling.³⁵⁻³⁹ PQQ can also serve as an antioxidant.⁴⁰ The importance of PQQ to mammalian health is evident when it is omitted from chemically defined diets, resulting in a wide range of systemic responses, including growth impairment, compromised immune responsiveness, and abnormal reproductive performance in mouse and rat experimental models.^{8,25,26,41,42} Furthermore, varying PQQ in highly

refined diets causes modulation in mitochondrial content, alters lipid metabolism, and reverses inhibition elicited by classical complex I inhibitors.^{39,41-43}

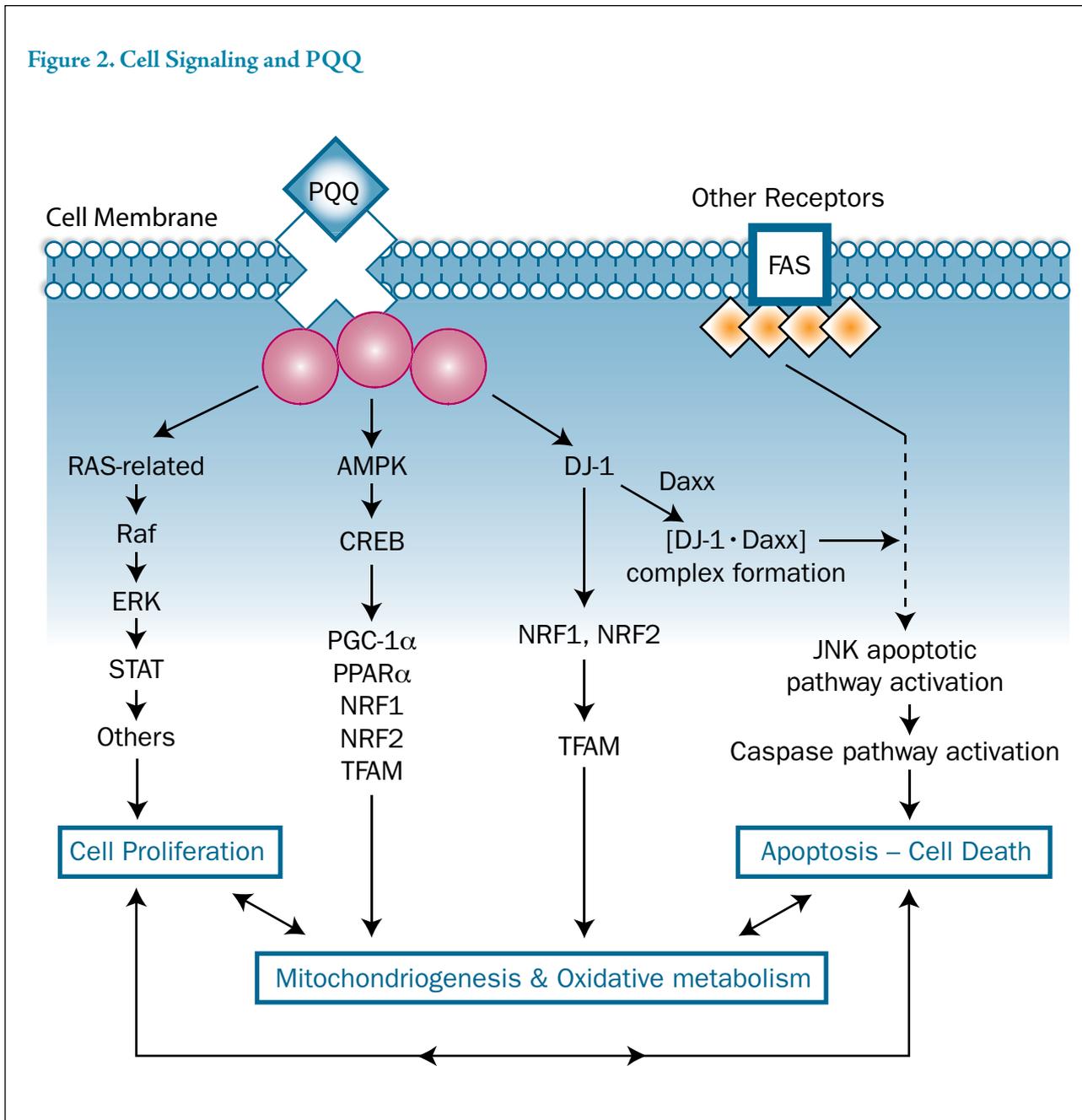
Improvements in mitochondrial respiratory control are potentially important to a number of health issues, ranging from increased longevity to improved energy utilization and protection from reactive oxygen species. Mitochondrial DNA depletion and mutations are associated with cardiomyopathy, developmental delays, and impaired neurological and mitochondrial function,⁴⁴ which further highlights the importance of optimal mitochondrial function for health and well-being. Regarding possible mechanisms of PQQ action (Figure 2), given that many mitochondrial-related

events are regulated by peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1 α) and nuclear respiratory factors, an interaction between PQQ and a PGC-1 α -related pathway seems a logical possibility. Indeed, such interactions have recently been reported.⁴⁴⁻⁴⁶

PGC-1 α is a transcriptional coactivator that regulates genes involved in energy metabolism.^{45,46} An interaction with this protein and its association with multiple transcription factors can provide a direct link between an external physiological stimulus and the regulation of mitochondrial biogenesis. PGC-1 α is also a major factor that regulates muscle fiber type determination and appears to be involved in controlling blood pressure, regulating cellular cholesterol homeostasis, and the development of obesity. Moreover, PGC-1 α is associated with a reduction in reactive oxygen species and protection against various mitochondrial toxins.⁴⁵



Figure 2. Cell Signaling and PQQ



In addition to interacting with PGC-1α, PQQ can also affect the activity of ras,³⁸ an oncogene important to signal transduction processes involved in growth and development. PQQ can stimulate activated c-Ha-ras-transformed NIH3T3 mouse fibroblasts,³³ resulting in increased cell proliferation. With regard to ras, activation usually results in changes in cell growth, differentiation, and survival. In addition to ras,

Kumazawa et al³³ have also observed that activation of ERK occurs in response to adding PQQ to cultured fibroblasts. ERK is one of the many protein kinases that functions in the ras-signaling pathway to activate other components of transcription (e.g., activators, co-activators, and transcription factors). Similarly, raf is depicted in Figure 2, because like ERK it is also part of the chain of events that progress from ras to eventual

activation of signal transducers and activators of transcription (STAT) factors that are essential to control of cell growth, survival, and differentiation. Regarding PGC-1 α -related cell signaling,^{8,39,41-43} activation starts with signals from 5' AMP-activated protein kinase (AMPK) or one of the many mitogen-activated protein (MAP) kinases that are linked to various cell surface receptors.^{44,45} Such kinases activate cAMP response element binding protein (CREB), which is a transcription factor that binds to certain DNA sequences called cAMP response elements. The authors recently reported³⁹ that CREB activation in combination with other transcription factors, such as nuclear respiratory factors 1 and 2 (NRF) and mitochondrial transcription factors (e.g., Tfam), leads to increased mitochondrial biogenesis observed with PQQ administration.⁴¹⁻⁴³

PQQ has been reported to influence the activity of DJ-1³⁷. DJ-1 is involved in cellular oxidative stress responses, and autosomal-recessive mutations in DJ-1 lead to Parkinson's disease. A possible role of an interaction between DJ-1 and PQQ may be to facilitate and fine-tune overall cellular regulation.³⁷ The cell signals for growth and energy utilization via mitochondria are in communication with apoptotic (programmed cell death) signals. DJ-1, most likely by associating with Daxx (a multifunctional protein associated with apoptotic events), is capable of modulating apoptosis by inactivating yet another cell signaling pathway, the so-called Janus kinase (JNK) cell-signaling pathway. An important feature of this interaction is potential "crosstalk" with STAT components and control of caspase activation/deactivation (cysteine-aspartic acid proteases). Caspases are associated with apoptosis or in pathological situations with cellular necrosis and inflammation. Accordingly, when all is taken together, an important feature of the interactions depicted in Figure 2 is the crosstalk between the signals, such as ras, DJ-1, and numerous kinases, that control proliferation, apoptosis, and mitochondrial biogenesis.

Regarding PQQ's role as an antioxidant, recent studies on the aroxyl radical-scavenging action of reduced PQQH₂ have shown PQQ exists in cells in a reduced form.⁴⁰ Like vitamin C, glutathione, vitamin E, and uric acid, PQQ can act as an antioxidant.⁸ As examples, Tsuchida et al⁴⁷ and Urakami et al⁴⁸ reported PQQ protects against acute liver damage induced by agents

such as carbon tetrachloride or endotoxin. Hamagishi et al⁴⁹ observed that PQQ administered (i.p.) at 10 or 30 mg/kg body weight causes a decrease in carrageenan-induced edema by 39- and 76 percent, respectively. It is also noteworthy that on a molar basis, PQQ is a better inhibitor of tissue oxidation in peritoneal cells than α -tocopherol and ascorbic acid, following initiation by zymosan, carrageenan, or N-formyl-methionyl-leucyl-phenylalanine, all of which provoke inflammatory responses.⁴⁴ From a mechanistic perspective, in addition to serving as an antioxidant, the effects of PQQ on genes, such as PGC-1 α , DJ-1, and genes in the ras family help explain many of the physiological and clinical functions ascribed to PQQ.

Clinical Implications

The following subsections briefly describe clinical implications of PQQ use. Although much of this work was conducted in animal models, current efforts in humans and human cell lines demonstrate important parallels.

Improvements in Reproduction, Early Development, Growth, and Immune Function

Nutritional studies indicate PQQ can serve as a growth factor and improves neonatal survival.^{8,31,41,42} In human fibroblast cultures, PQQ enhances cell growth and proliferation when added to cell cultures.^{38,50} Signs of PQQ deprivation include friable skin, evidence of hemorrhage and diverticuli, and reduction in general fitness. The growth-related observations are novel in that adding 100-200 μ g PQQ/kg to purified diets improves growth, development, and reproductive parameters in rodent models.^{8,30} For perspective, the animal requirements for folic acid or for biotin range from 200-500 μ g/kg diet, respectively. These effects are similar to the improvements when more complex diets are fed (i.e., made of less refined ingredients).^{30,31} Moreover, in female mice and rats fed PQQ-deficient diets, fertility is decreased (fewer successful pregnancies and smaller litter size)^{30,31} compared to mice or rats fed PQQ-supplemented diets.

PQQ deprivation also results in defects in immune function and reduction in interleukin-2 (IL-2) levels. There is loss of B- and T-cell sensitivity to mitogens. The body normally produces IL-2 during an

immune response. IL-2 is necessary for the development of T-cell immunologic memory, one of the unique characteristics of the immune system. Maximizing sensitivity of B- and T-cells to mitogens is achieved in mice when as little as 1 nmol PQQ is added per gram of diet, about 100-400 µg PQQ per day in human equivalents.^{30,31}

PQQ and Neuroprotection

Neuronal cell death in experimental models of stroke and spinal cord injury is attenuated by PQQ.^{31,32,51-54} PQQ has been demonstrated to protect the redox modulatory site of N-methyl-d-aspartic acid (NMDA) receptors.^{36,51-59} Agents that protect NMDA-receptor function are often neuroprotective in experimental stroke and spinal cord injury models. In this regard, intraperitoneal administration of PQQ effectively promotes the functional recovery of spinal cord injury in rats after hemi-transection.³⁶ Protection is preceded by a decrease in inducible nitric oxide synthase (iNOS) mRNA. Nitric oxide is implicated in NMDA receptor-mediated neurotoxicity. Administration of PQQ decreased lesion size and increased axon density associated with the lesion area. Furthermore, recent studies suggest PQQ protects against secondary damage by reducing iNOS expression following a primary physical injury to the spinal cord. Peroxynitrite is a potential byproduct of abnormally high nitric oxide (NO) or cellular hydrogen peroxide levels. The demonstration that iNOS expression is reduced is in part a validation of previous work showing that PQQ treatment suppresses peroxynitrite formation.⁵⁶ Moreover, PQQ's ability to affect the oxidative status of DJ-1 adds an additional dimension.³⁷ As has been noted previously, the expression level and oxidation status of DJ-1 have been shown to play a role in antioxidative stress reactions important to neurological function. These findings add to the initial observations by Jensen et al⁵⁴ that PQQ effectively reduces infarct size in an experimental model of cerebral hypoxia/ischemia. PQQ administered i.p. at 10-15 mg/kg body weight in rats was effective in reducing cerebral infarct volumes measured 72 hours or more after a neurovascular insult. Three hours after ischemia a dose of 3 mg/kg significantly reduces infarct volume compared to vehicle-treated animals. These data indicate PQQ may be a useful neuroprotectant in stroke therapy.

Even at a more subtle level, PQQ exposure can affect learning ability and memory function in rats.⁶⁰ Rats fed a PQQ-supplemented diet demonstrate improved learning using the Morris water maze test as an index. Rats were fed 20 mg PQQ, 300 mg coenzyme Q10 (CoQ10), 200 mg R,R,R- α -tocopherol, or 20 mg PQQ + 300 mg CoQ10/kg body weight/day for nine weeks (from age four weeks). Each rat was subjected to hyperoxia as the oxidative stress (using a 100% oxygen chamber) for 48 hours. Those fed PQQ-supplemented diets were protected from a memory deficit that was apparent in controls not fed PQQ. As a novel control, rats fed vitamin E-supplemented and -deficient diets were tested. Vitamin E-deficient rats fed PQQ and/or CoQ10 demonstrated improved learning function. In addition, longer-term memory function was maintained independently by PQQ, but not by CoQ10 supplementation. Thus, PQQ seems potentially effective in sustaining learning functions during oxidative stress, independent of and in a manner different from that of vitamin E.

PQQ and Cardiac Function

PQQ is useful in models of cardiac ischemia.^{43,61,62} PQQ confers resistance to acute oxidative stress in freshly isolated cardiomyocytes.⁶¹ Both oxidative damage and mitochondrial membrane potential depolarization (induced by hydrogen peroxide) are significantly reduced by preincubation with PQQ. Moreover, in whole animal models of damage due to cardiac ischemia and reperfusion, PQQ results in less cardiac damage, higher left ventricle pressures, and fewer ventricular fibrillation episodes, if given i.p. 30 minutes before occlusion.⁶¹ In rodent models of cardiac ischemia, PQQ at doses ranging from 5-20 mg/kg administered i.p. was inversely related to infarct size. In the same tests, PQQ was superior to metoprolol in protecting mitochondria from ischemia/reperfusion oxidative damage.⁴³

Side Effects and Toxicity

Safety studies for PQQ in humans have been conducted in preparation for several human use patents.^{63,64} PQQ was administered at 20 or 60 mg/day for four weeks to two groups (10 each) of healthy adults given either a PQQ supplement or a placebo. These studies were double-blinded and conducted at two different

commercial drug-testing facilities: the New Drug Clinical Center, Fukuhara Clinic, Eniwa, Hokkaido, Japan and Cronova Co., Ltd., Suminoeku, Osaka, Japan. No adverse effects were observed in standard clinical tests at either dose (e.g., glucose, triglycerides, and various lipoprotein fractions). Functional tests for liver toxicity were also normal (e.g., aspartate aminotransferase and serum glutamic oxaloacetic transaminase). At 60 mg PQQ daily, the amounts of urinary N-acetyl- β -(D)-glucosaminidase activity were also within the normal range. N-acetyl-glucosaminidase is a renal hydrolytic enzyme located primarily in the lysosomal fraction of the renal tubular cell. Abnormal changes in renal tubular function or damage results in its elevation in urine.⁶⁵

Single-dose oral toxicity tests in rats were performed in compliance with Good Laboratory Practice (GLP). The single-dose oral toxicity tests indicated the approximate lethal dose of PQQ is less than 1,000 mg/kg body weight of rats, but higher than 500 mg/kg.

Post-mortem pathological examinations of test rats suggest the kidney as the principal target organ for acute effects of PQQ. In part, this is a validation of an earlier published toxicology study⁶⁶ in which PQQ was administered intraperitoneally to rats at a dose of 11-12 mg/kg body weight. Signs of renal tubular damage and inflammation were observed. When lower doses were used, however, no treatment effects or obvious pathological signs were observed. Likewise, in a 90-day repeated dose study in which PQQ was administered to rats by oral gavage (3, 20, or 100 mg PQQ/kg body weight) no adverse effects were observed. Moreover, at oral dosage levels from 250-2,000 mg PQQ/kg in mice, an examination for micronucleus induction in red blood cells showed no effects. Lastly, the results from a battery of genotoxicity tests *in vitro* (the Ames, micronucleus, and chromosomal aberration tests) were negative, i.e., PQQ did not cause clastogenic toxicity (chromosome breaks, rearrangements and changes in chromosomal number).

In summary, these observations taken together suggest there is no evidence of acute side effects or overt toxicity from consuming PQQ in amounts up to 60 mg per day for humans or several hundred mg per kg of diet fed to animals.

Dosage

Regarding typical exposures of free PQQ, as noted above, the amount for humans is estimated to vary from 100-400 μ g daily,^{11,25,28,67} about the same as the daily nutritional recommendations for biotin and folic acid, respectively. However, PQQ easily forms condensation products upon interaction with amino acids,²⁶ complicating the precision of such estimates. The primary condensation products are imidazolopyrroloquinoline (IPQ) and imidazolopyrroloquinoline derivatives with attached amino acid side chains as part of the chemical structure. For example, only about 15 percent of the PQQ is present in free form in biological fluids such as human milk, while 85 percent is present as IPQ and derivatives.²⁶ Thus, it is not unreasonable to assume that for humans the total exposure to PQQ derivatives may be as much as 1-2 mg per day. This amount is in the range that clearly influences optimization of growth and health in animal models.⁸ In the case of human milk, PQQ amounts to 1-2 μ g PQQ/g of milk solid, which is also similar to the PQQ concentrations reported for bovine milk.²⁶ It is important to note that PQQ appears readily absorbed. Smidt et al⁶⁸ determined that the apparent absorption of an oral dose of ¹⁴C-PQQ ranges from 20-80 percent when administered to adult mice in the fed state. The percentages were estimated from the amount of radioactivity present in urine and tissues 24 hours after administration.

Conclusions

The observation that increased mitochondrial biogenesis and antioxidant functions may be healthful features of PQQ supplementation opens the doors for both therapeutic applications and possible use as an ergogenic aid. Having normal mitochondrial function is essential to a broad range of health and disease relationships; thus, the need for continuing research that examines the efficacy and use of PQQ is compelling. PQQ derivatives are widely distributed in tissues and biological fluids at concentrations that may be sustained by typical dietary exposures. Given the range of functions and apparent survival benefits (e.g., improved reproductive performance), it is reasonable to suggest that PQQ may play a fundamental role in metabolism.

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Corrections

Altern Med Rev 2009;14(2):143.

Figure 2. Meriva should read Meriva curcuminoids, curcumin should read curcuminoids.

Paragraph 3: "One small unpublished..."

Should read: "One small unpublished, single-dose trial demonstrated 450 mg of Meriva curcuminoids complexed with phosphatidylcholine was absorbed as efficiently as 4 g unbound *Curcuma longa* (95% curcumin), reflecting a significant increase in bioavailability for Meriva complex (Figure 2).15

Effects of Oral Supplementation with Pyrroloquinoline Quinone on Stress, Fatigue, and Sleep

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ABSTRACT:

Seventeen adult male and female subjects participated in a clinical trial using an open-label trial to evaluate the effectiveness of pyrroloquinoline quinone (PQQ) on stress, fatigue, quality of life and sleep. They ingested 20 mg of PQQ daily for 8 weeks. Changes in stress, fatigue, quality of life measures and sleep were evaluated using various inventories and questionnaires. For example, the results of the Profile of Mood States-Short Form revealed that all six measures of vigor, fatigue, tension-anxiety, depression, anger-hostility and confusion were significantly improved following PQQ administration compared with scores for those measures before administration of PQQ. Measures for quality of life, appetite, sleep, obsession and pain, also improved significantly. The results of the Oguri-Shirakawa-Azumi Sleep Inventory (Middle Aged and Aged version) showed significant improvement in sleepiness at awakening, sleep onset and maintenance, and sleep duration. For validation, the Pittsburgh Sleep Quality Index Japanese version also showed significant improvement in sleep-related behavior. Furthermore, the changes in these global scores were correlated with

changes in the cortisol awakening response ($R = -0.55$), i.e. the effects of PQQ on improvement of sleep quality are supported by a biomarker.

Keywords: Pyrroloquinoline quinone, stress, fatigue, quality of life, sleep

INTRODUCTION:

Pyrroloquinoline quinone (PQQ) is an organic molecule discovered in bacteria in 1979 as the third redox coenzyme following nicotinamides (pyridine nucleotides) and flavines PQQ is water-soluble, having 3 carboxyl groups, a quinone, and chemical properties similar to the combined attributes of ascorbic acid, riboflavin, and vitamin B₆. PQQ is present in various vegetables and beverages, especially in tea, natto (fermented soybeans), and fruits [1, 2], and is also found in the human tissues and fluids, especially breast milk. In animal studies, PQQ as administration influences anti-oxidative capacity, nerve growth factor enhancement and mitochondrial biogenesis [3-7]. Based on such data, we conducted studies that indicate PQQ administration has a positive influence on brain functions, especially short-term memory and attention in elderly healthy volunteers [8]. Accordingly, and to add to our previous observations, we focus herein on mental stress, fatigue, quality of life (QOL) and sleep quality. Adult male and female office workers who complained of fatigue and/or disordered sleep were used as subjects.

SUBJECTS AND METHODS:

Test substance. The test substance was provided in the form of a hard capsule containing 20 mg of pyrroloquinoline quinone Na₂ salt (BioPQQ™) manufactured by Mitsubishi Gas Chemical Co., Inc. (Tokyo, Japan). A capsule was ingested with a cup of water once a day after breakfast. The time of ingestion and the number of capsules ingested were recorded in a diary. The duration of the study was 8 weeks. The dose of 20 mg of PQQ per day was based on our previous clinical study [8].

Study design. The study followed an open-label trial. The effectiveness of PQQ was assessed by comparing the evaluation measures before and after PQQ administration. Primary evaluations consisted of four methods of subjective self-reporting were adopted for evaluating stress, fatigue, QOL and sleep quality. In addition, the increase of cortisol secretion called the cortisol awakening response (CAR) was measured. We adopted CAR as an objective method of evaluation. In general, job stress or general life stress raises the cortisol following

awakening, while fatigue, burnout or exhaustion reduces that [9]. With respect to sleep, some studies report that high CAR is associated with healthy sleep [10, 11]

Subjects. Seventeen volunteers were selected from adult male and female workers with a diagnosed sleep disorder or complaint and fatigue using as selection criteria the Athens Insomnia Scale (AIS), a scale of insomnia [12], and the Profile of Mood States-Short Form (POMS-S) [13].

Selection criteria were: (1) men and women aged from 20 to 60; (2) those employed full-time; (3) those who scored over 6 points on the AIS at the examination before enrollment; and (4) those who scored over 50 points in the T score of fatigue and below 50 points in the T score of vigor on the POMS-S at the examination before enrollment. Exclusion criteria, discontinuance criteria, dropout criteria, and restrictions were listed in Supporting Information.

The study was conducted in compliance with the “Declaration of Helsinki” and “The Ethical Guideline for Epidemiological Study” (issued in 2004 by the Ministry of Education, Culture, Sports, Science and Technology, and the Ministry of Health, Labour and Welfare). The study (Protocol No.21584) was approved by the clinical study review committee of the Miho Clinic (Tokyo, Japan) on the basis of the protocol and information on the test substance on July 17, 2009. Actual study was done in Ueno Clinic (Tokyo, Japan). The details of this study were explained to the subjects before the start of the study and informed consent given by free will was obtained from each subject.

Study timelines. The subjects visited Ueno Clinic for the first examination. Prior to PQQ intervention, the subjects filled out the POMS-S inventory, the life event survey, the Pittsburgh Sleep Quality Index Japanese version (PSQI-J) and the QOL questionnaire and underwent physical examination (anthropometry, blood pressure and heart rate) and blood and urine tests. Following selections, subjects were next kept overnight in a comfortable setting so that saliva samples could be collected 30 min after awakening to determine the cortisol levels in saliva. The subjects then filled out the Oguri-Shirahata-Azumi Sleep Inventory (Middle Aged and Aged version) (OSA-MA) soon after awakening to evaluate sleep quality during the past one week.

The subjects were then instructed to ingest the test substance and visit the clinic on the weekends of the 1st (1w), 2nd (2w), 4th (4w) and 8th week (8w) after the initiation of supplementation. On the clinic examination days, they filled out the QOL questionnaire every 4 weeks and the POMS-S every 2 weeks. The POMS-S on the 6th weekend (6w) was filled out

at home. An examination of their general condition, including physical examination as well as blood and urine tests, was conducted every 4 weeks. On weekend days, the OSA-MA was filled out soon after awakening at home. Saliva samples were collected soon after and 30 min after awakening on the latest weekday before the clinic examination day of the weekends of 4w and 8w. On the examination day, the collected saliva samples were brought to the clinic. The test schedule is shown in Table S1 (see Supporting Information). The duration of this study was from July to November, 2009.

Evaluation of POMS-S and QOL. The effectiveness of PQQ was evaluated using the POMS-S and the QOL Questionnaire to measure the changes at each time point after ingestion and also by the results of intergroup comparison between the values prior to supplementation (the baseline values) and the values at each time point after supplementation.

The POMS Japanese version is a questionnaire comprising 65 questions about six components: tension-anxiety, depression, anger-hostility, vigor, fatigue and confusion [14]. The POMS-S shortens the number of questions from 65 to 30, for ease of use [13]. Despite this, it is highly reliable and produces similar results to the POMS. For each question, the subjects select one of five answers ranging from “not at all” (0 points) to “very frequently” (4 points), according to how many times the mood in question occurred in the past 7 days. All 30 questions are divided into six components with five questions each, and the total points for each component are calculated and designated the raw score. After the raw score is corrected for age and gender, the standardized score (T score) is calculated. The higher score for vigor indicates the better condition, while the lower score for the other five components indicates the better condition.

QOL was evaluated by Nagata’s QOL Questionnaire [15, 16], scoring measures for appetite, sleep, excretion, urination, exercise, obsession, pain, sexual life, social life, happiness, family life, and fullness and satisfaction in the overall daily life. For each question, the subjects select one of five to twelve answers ranging from “best” (1 points) to “worst” (5 points). In cases of multiple answers, the maximum point score was adapted. For all measures, the lower score indicates the better condition.

Evaluation of sleep. Two subjective methods were employed to enhance the reliability of the results of evaluation: the OSA Sleep Inventory and the PSQI-J, which measured changes at respective examination time points after the start of the ingestion of PQQ, allowing intergroup comparison between the values before ingestion (baseline values) and those after ingestion.

The OSA-MA consists of 16 questions divided into five factors: Factor I (sleepiness at awakening), Factor II (sleep onset and maintenance), Factor III (nightmare), Factor IV (recovery from fatigue) and Factor V (sleep duration)[17]. The subjects select, soon after awakening in the morning, the best answer to each question from the following: “remarkably”, “modestly”, “slightly” and “not at all”. The answers are converted into Zc points and the average point score for each factor is calculated as the evaluation score of that factor (Factor I: 0-32.3, Factor II: 0-29.6, Factor III: 0-29.5, Factor IV: 0-32.7, Factor V: 0-33.5). For all questions, the higher score means the better condition.

The PSQI is a questionnaire developed in the US for screening of sleep disturbance, composed of 18 questions for the seven components of sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication and daytime dysfunction. In this study, the PSQI-J was used [18-20]. The PSQI global score (from 0 to 21) was obtained by totaling the scores of the seven components (0–3). Higher global scores show more disturbed sleep, with a total score of more than six indicating sleep disturbance. The global score and the scores of the seven components were calculated.

Measurement of salivary cortisol. In the present study, the increase of salivary cortisol level from soon after awakening to 30 min after awakening was defined as CAR and used to determine the amount of cortisol secretion non-invasively [9].

Samples were collected on the latest weekday before the examination day and on the examination day (weekend) at 0w (before PQQ ingestion), 4w and at 8w by using the saliva sampling tube (Salisoft) (Assist Co., Tokyo, Japan). The times of awakening and sample collection were recorded. Until the completion of sample collection, eating, drinking, smoking and tooth brushing were not permitted. The saliva samples collected were kept in a home refrigerator and brought in a cooler bag with cooling agent to the clinic on examination day. The concentration of salivary cortisol was determined by means of the salivary cortisol EIA kit (Salimetrics) of expanded range and high sensitivity.

Safety evaluation. Adverse events were recorded for subjects who had taken PQQ at least once. Any undesirable subjective or objective symptom was regarded as an adverse event and appropriate treatment was administered if necessary, with recording of details of symptoms and findings, the date of occurrence and the date of resolution. The seriousness of the event, the details of treatment (if treated), the outcome and the cause and effect relationship to PQQ were reviewed and recorded on the case report form.

The safety of PQQ was evaluated based on the adverse events (subjective symptoms, objective findings and abnormal changes in the measured values) that were judged to be possibly related to PQQ.

Statistical analysis. All measured values and changes were expressed as mean \pm SD. If the analysis of variance by repeated measure revealed a statistically significant difference in the measured value before and after ingestion, the baseline value and the measured values at each examination time after ingestion were compared by the single sample t-test for the POMS-S and OSA-MA. For PSQI-J and QOL, the values were compared by the Wilcoxon signed rank test. The multiple comparisons were made by the Holm's method. For the physical examination values, the changes at each time point after ingestion from the baseline values were evaluated by the single sample t-test. The two-sided level of significance was 5%.

RESULTS:

Subjects: background information and physical examination.

The seventeen subjects (seven men and ten women) were 38.1 ± 7.4 years old. The results of physical examination are presented in Table 1. There was no discontinuance or dropout during the study period. At the time of their selection, the scale for sleeplessness by the AIS was 12.6 ± 2.8 and the global score by the PSQI-J was 10.0 ± 1.9 , demonstrating disordered sleep. The T score of [Vitality] in the POMS-S was 35.7 ± 4.6 and that of [Fatigue] was 71.8 ± 5.1 , demonstrating stress and fatigue. During the study, none of the subjects had any major changes in employment and working hours or stressful events such as changes in home environment, diseases and birth or death of close relatives.

Table 1. Results of physical examination

Measurement	Unit	0w	4w	8w
Height	cm	163.75 ± 9.47		
Weight	kg	58.26 ± 10.33	58.35 ± 10.39	58.66 ± 10.60
BMI	kg/m ²	21.54 ± 1.93	21.58 ± 1.93	21.68 ± 2.03
Systolic blood pressure	mmHg	112.5 ± 12.2	108.6 ± 11.4 *	113.0 ± 11.1
Diastolic blood pressure	mmHg	66.8 ± 7.2	65.5 ± 6.1	67.6 ± 4.3
Heart Rate	bpm	70.1 ± 7.2	68.1 ± 10.1	66.9 ± 6.8

$n = 17$ paired t-test * : $p < 0.05$

Effects of PQQ on stress, fatigue and QOL.

The results of POMS-S to evaluate stress and fatigue are presented in Figure 1. The analysis of variance by repeated measures revealed that duration-dependent significant decreases were observed in five components, except vigor, with PQQ ingestion.

The scores of mood states before PQQ ingestion and after eight-weeks of PQQ ingestion are shown in Figure 2. After 8w, the T score of vigor increased, while those of other five components decreased. These results indicated improvements in stress and fatigue with PQQ.

The results of the evaluation of QOL are shown in Table 2. The four measures of (Appetite, Sleep, Obsession and Pain) significantly declined, indicating significant improvement with PQQ. The three measures of (Fullness of social life, Happiness and fullness in the family life, and Fullness and satisfaction in the overall daily life) showed tended to decline (i.e. improve).

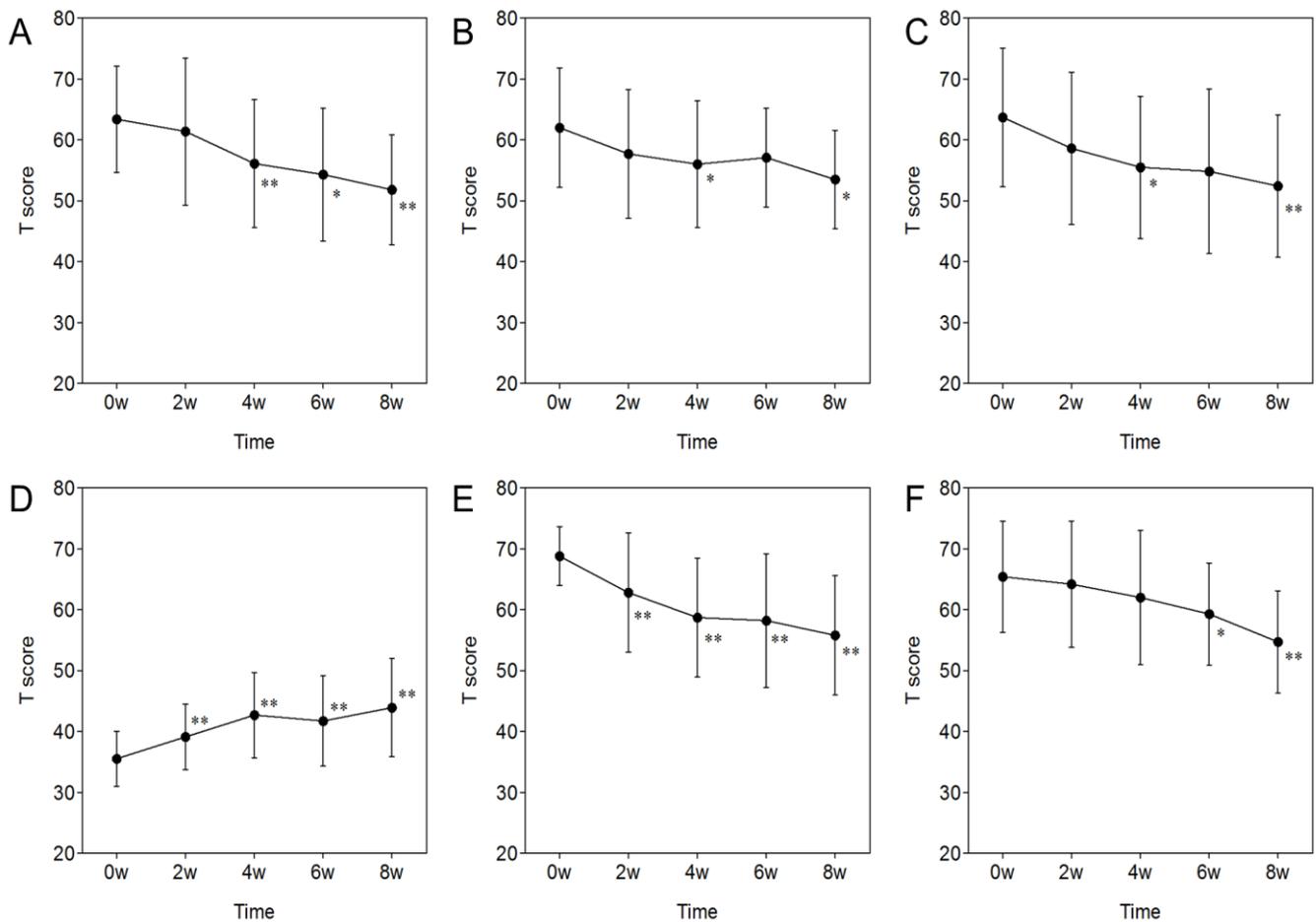


Figure 1. Effects of PQQ supplementation on POMS-S scores of (A) Tension-Anxiety, (B) Depression, (C) Anger-Hostility, (D) Vigor, (E) Fatigue, and (F) Confusion ($n = 17$). Vertical bars indicate standard deviation. (paired t-test ** : $p < 0.01$, * : $p < 0.05$)

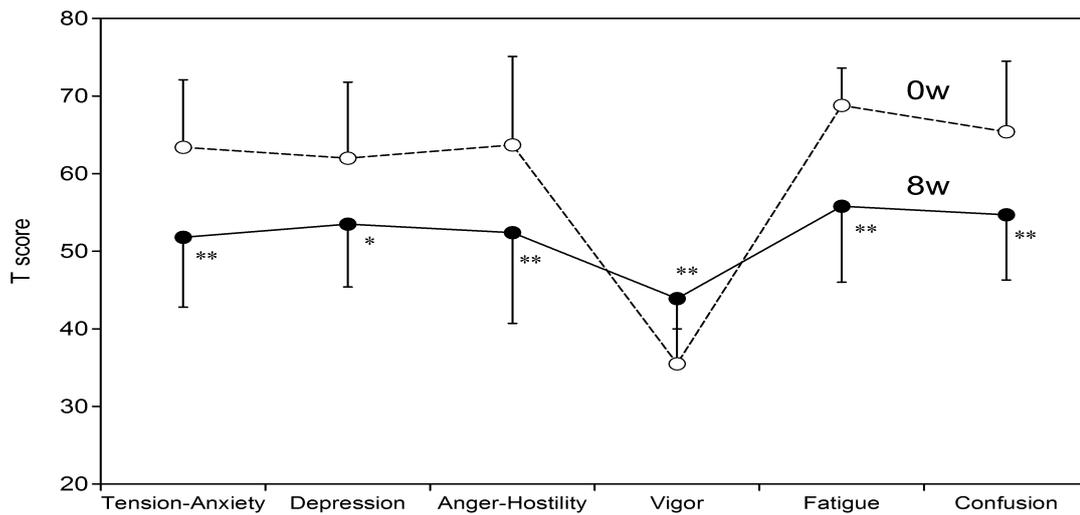


Figure 2. Effects of PQQ supplementation on POMS-S scores ($n = 17$). Vertical bars indicate standard deviation. (paired t-test **: $p < 0.01$, * : $p < 0.05$).

Table 2. Effects of PQQ supplementation on QOL scores

Measurement	0w	4w	8w
Appetite	3.5±0.9	2.5±0.9 **	2.3±0.7 **
Sleep	4.2±0.4	3.5±0.9 *	3.0±0.9 **
Excretion	3.0±0.8	2.9±0.9	2.6±1.1
Urination	3.4±0.9	2.8±1.1	2.9±0.8
Exercise	3.4±1.3	3.6±1.1	3.6±1.0
Obsession	3.8±0.8	3.2±1.3 *	2.5±1.1 **
Pain	3.6±0.8	3.3±0.8 *	2.9±1.1 *
Sexual life	3.8±1.0	3.6±1.2	3.6±1.2
Fullness of the patient's social life	3.0±0.7	2.6±0.6	2.5±0.5
Happiness and fullness in family life	2.4±1.1	2.2±0.9	2.0±1.0
Fulfillment and satisfaction in overall daily life	3.1±0.4	2.8±0.6	2.7±0.6

$n = 17$, Wilcoxon signed rank test (multiple comparison by Holm's test)

** : $p < 0.01$, * : $p < 0.05$

Effects of PQQ on sleep.

The results of the OSA-MA are shown in Figure 3. PQQ ingestion caused significant duration-dependent improvements in four of the five factors except for Factor III (nightmare).

Table 3 shows the results of the PSQI-J. The PSQI-J global score went down depending on the duration of PQQ ingestion, as did individual components such as sleep quality, sleep latency, sleep duration and daytime dysfunction.

Finally, to investigate the relation of CAR to sleep, the correlation between CAR and the global score of PSQI-J was assessed. The ten subjects with CAR values below zero at 0w and 8w were excluded as “non-compliant to protocol”, i.e. collection of saliva beyond the 30 minutes post-sleep cut-off [9, 21, 22] (Table S2, see Supporting Information). In the analyses of seven compliant subjects, the 8-week changes in PSQI scores were correlated with changes in CAR ($R = -0.55$) (Figure 4).

Table 3. Effects of PQQ supplementation on sleep (PSQI-J scores)

Component	0w	4w	8w
PSQI-J global score	10.0±1.9	8.2±2.1 **	6.4±2.0 **
Sleep quality	2.2±0.4	1.6±0.5 **	1.5±0.7 **
Sleep latency	2.1±0.9	1.8±0.8	1.4±0.9 **
Sleep duration	2.4±0.6	2.1±0.6 *	1.9±0.6 **
Habitual sleep efficiency	0.5±0.6	0.2±0.4	0.1±0.2
Sleep disturbance	1.1±0.3	1.1±0.7	0.8±0.6
Use of sleeping medication	0.0±0.0	0.2±0.7	0.0±0.0
Daytime dysfunction	1.8±0.8	1.4±0.5 *	0.9±0.7 **

$n = 17$, Wilcoxon signed rank test (multiple comparison by Holm's test)** : $p < 0.01$, * : $p < 0.05$

Safety.

1. Subjective symptoms and objective symptoms

During ingestion of PQQ, six events occurred in five subjects, but were not judged as related to PQQ. These events were the common cold in four subjects including one complaint of abdominal pain and a slight fever in another subject. Symptoms were minor and transient despite continued ingestion of PQQ.

2. Results of physical examination

Table 1 shows the changes of mean values and standard deviations obtained from the physical examination. They were judged that all of the changes were clinically negligible. There were no adverse events. Systolic blood pressure was also reduced significantly at 4w, a clinically significant positive effect.

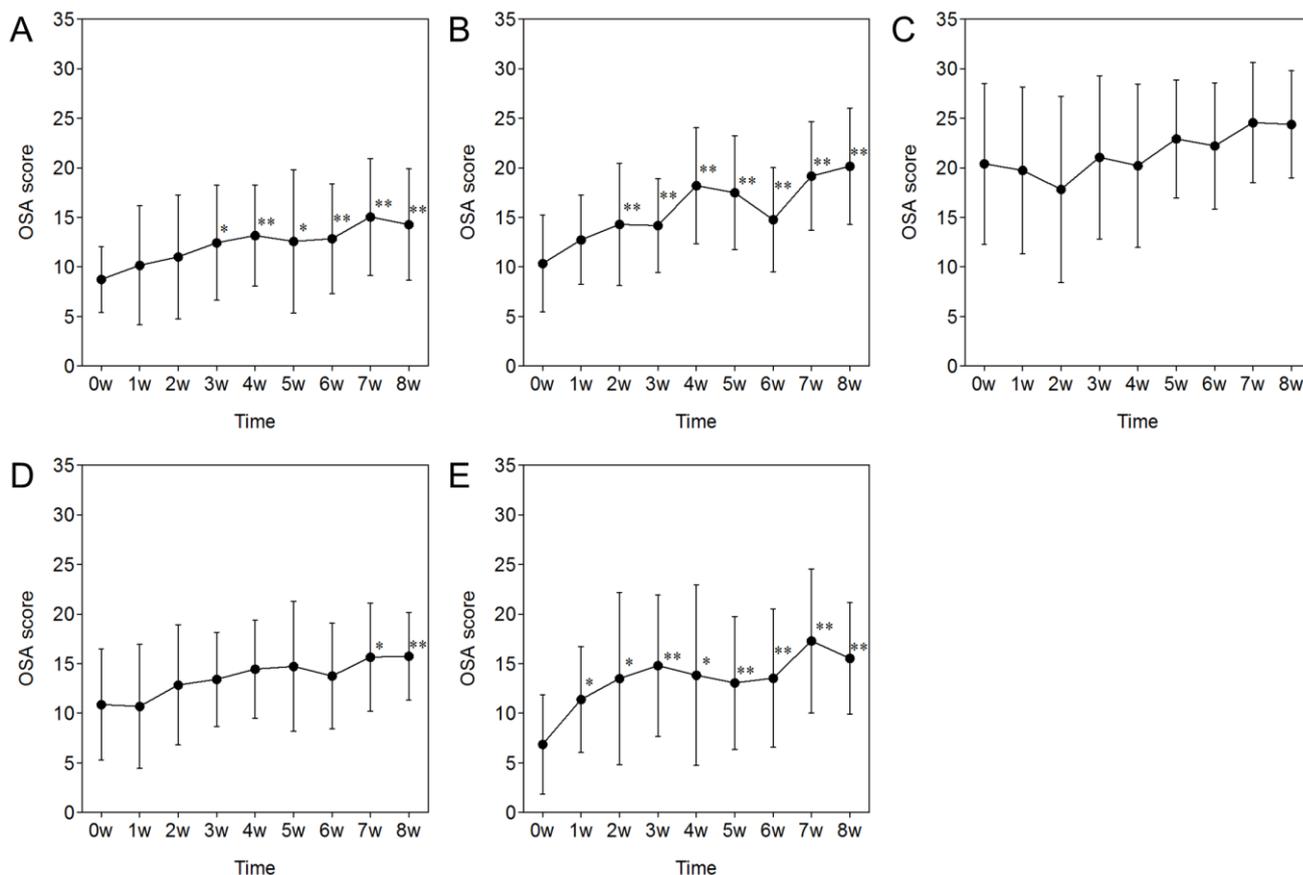


Figure 3. Effects of PQQ supplementation on OSA-MA scores of (A) Factor I (sleepiness at awakening), (B) Factor II (sleep onset and maintenance), (C) Factor III (nightmare), (D) Factor IV (recovery from fatigue), and (E) Factor V (sleep duration) ($n = 17$). Vertical bars indicate standard deviation. (paired t-test ** : $p < 0.01$, * : $p < 0.05$).

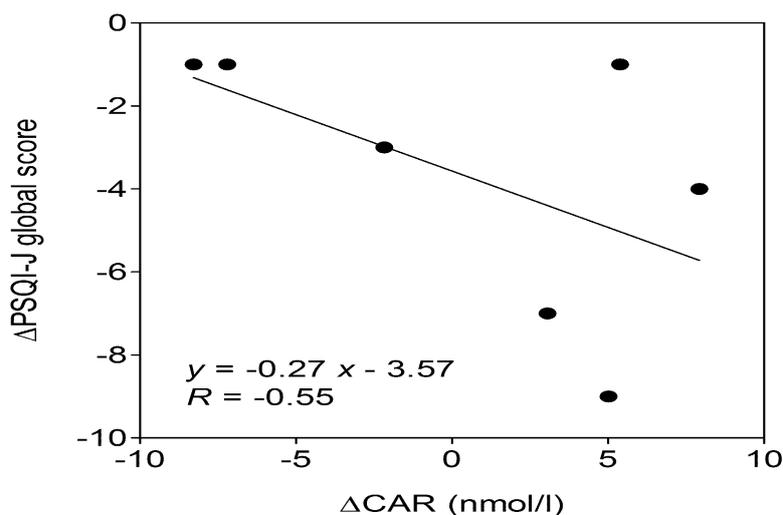


Figure 4. The correlation between changes in weekday CAR and changes in PSQI-J global score after 8 weeks of ingestion ($n = 7$). The line represents a linear regression ($y = -0.27x - 3.57$, $R = -0.55$). Raw data represents Table S4.

DISCUSSION:

This was the first human study on the effectiveness of oral PQQ supplementation focusing on stress, fatigue, sleep and QOL. Both the OSA-MA and PSQI-J demonstrated significantly improved sleep quality. The concurrent results from two different evaluation methods strongly supported study validity. The results of subjective evaluations by the POMS-S, OSA-MA and PSQI-J suggest that PQQ supplementation also improved sleep, leading to reduced negative mental states, relief of fatigue and rise of positive mood (Table 2).

The scores of the five components, except vigor, in the POMS-S evaluation continued to fall and scores for vigor continued to rise during 8 weeks (Figure 1). Scores of most factors of the OSA-MA showed an upward trend during 8 weeks (Figure 3). These data suggest that PQQ improved not only fatigue but also disordered sleep and QOL.

To examine the physiological basis of this action, we also measured CAR as a biomarker. It has been reported that sleep quality is closely related to CAR [11]. Moreover, in the comparison between primary insomnia patients and healthy volunteers, Backhaus et al. [10] observed that the average cortisol level of the former at awakening was significantly lower than that of the latter. On the other hand, lower PSQI-J global score indicates better sleep quality. In the analysis of our compliant subjects ($n = 7$), a correlation was observed between changes in PSQI-J global score and changes in CAR after 8-weeks of PQQ ingestion (Figure 4 and Table S3, see Supporting Information). These findings suggest that when normal sleep is recovered with PQQ and cortisol secretion due to sleep disturbance is repressed, which occurs with a normal or improved awakening response.

This study is the first human clinical trial on the effect of PQQ on stress, fatigue, and sleep, to better reflect the primary outcome measurements. The results of this trial are therefore, encouraging and should be extended in a placebo-controlled study with a larger study population. The anti-oxidative capacity or mitochondrial biogenesis function of PQQ might be responsible for the outcomes in this study. However, the elucidation of the mode of action is needed in further study.

Conclusions: In conclusion, PQQ supplementation seems associated with improved sleep quality and duration. Mood also was improved by diminishing feelings of fatigue, and improved QOL on measures of appetite, sleep, pain and obsession. The improvement of the PSQI-J global score correlated with changes in CAR. These data indicate that PQQ can be regarded as a potentially useful dietary supplement as it may relate to improving fatigue. Importantly, no health issues were associated with PQQ supplementation.

Competing interests: The authors have no financial interests or conflicts of interest.

Author's contributions: All authors contributed to this study.

Abbreviations: AIS, Athens Insomnia Scale; CAR, cortisol awakening response; OSA-MA, Oguri-Shirakawa-Azumi Sleep Inventory (Middle Aged and Aged version); POMS-S, Profile of Mood States-Short Form; PQQ, pyrroloquinoline quinone; PSQI-J, Pittsburgh Sleep Quality Index Japanese version; QOL, quality of life;

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Supporting Information

Subjects and Methods

Exclusion criteria

1) Those who took routinely the foods or health foods compounded with the active constituent of this test food (PQQ)

2) Those who had regularly any medical treatment (or corresponding treatment) or took medicines or health foods for the purpose of improving sleep, fatigue and stress

3) Those who were engaged to works on a day-night shift or physical labors such as transportation of heavy goods and were working on an irregular schedule

4) Those who did not follow the prescribed procedures for various examinations performed during this study (such as saliva sampling soon after awakening or filling in questionnaires)

5) Those who had diseases under treatment or to be treated, including insomnia

6) Those who had the anamnesis of serious diseases such as diabetes mellitus, hepatic disorders, renal disorders, hypertension, cardiovascular disorders and abnormal glucose tolerance

7) Those who were judged inadequate as the subject from the results of laboratory tests and physical examination performed before ingestion

8) Those who might possibly show allergic reaction to the test food (PQQ or other ingredients)

9) Those who expected to become pregnant during the study and were pregnant or breast-feeding

10) Those who had been already enrolled in another clinical study when they were recruited to this study

11) Those who were judged inadequate as the substance by the principal investigator of this study

Discontinuance criteria

The principal investigator could discontinue the study for the subject, when the principal investigator judged that the subject in question fell under the following items, and it was treated as a discontinuance case.

1) When the subject might threaten the safety of other subjects

2) When it was difficult to continue this study due to occurrence of any serious clinical abnormality or accident

- 3) When any critical or continuous deviation from the protocol was found during the study
- 4) When the principal investigator decided to discontinue this study

Dropout criteria

When the subject withdrew from the study due to the personal reason or intention after the subject had agreed to enroll in this study, the principal investigator discontinued soon this study for the subject in question and treated it as a dropout case.

Restrictions

During the study, every subject had to comply with the following restrictions (including the notices to be followed till the day before examination and those on the day of examination).

- 1) The lifestyle before the enrollment in this study (eating, drinking, exercise, sleep, smoking, etc.) should be almost kept during the study. Excessive exercise, drinking and eating should be avoided.
- 2) When any medicine was taken, its name and amount should be recorded on the diary.
- 3) The test food should be taken every day in the prescribed amount.
- 4) The diary should be kept every day.
- 5) The measures to relieve stress should be taken within the ordinary range.
- 6) On the day before examination, alcohol should not be taken, and hard exercise should be avoided.
- 7) On the day before visiting the clinical for examination, eating and drinking should be prohibited after 24 o'clock, except drinking water or tepid water.
- 8) On the day of examination, the subjects should take the prescribed breakfast and thereafter should fast to the end of examination (water or tepid water is accessible).
- 9) The subjects should fill in the OSA-MA and collect the saliva sample before the time limit soon after awakening on the appointed day for examination. On the day of saliva sampling, eating, drinking, smoking and tooth brushing should not be permitted before the finish of saliva sampling 30 min after awakening.

Table S1. Test schedule and test item

Test schedule Test item		Before ingestion		Duration of ingestion								
		1	2	w	w	w	w	w	w	w	w	
Hospital visiting (holiday)												
Selection of subjects												
Lifestyle questionnaire												
AIS												
Physical examination												
Blood and urine tests												
Sleep	OSA-MA											
	PSQI-J											
	CAR											
Stress	POMS											
Fatigue	Life-event survey											
QOL questionnaire												
Ingestion of test substance				→								
Record in a diary				→								

*¹: Pre-enrollment examination

*²: Pre-ingestion examination

Table S2. Effects of PQQ supplementation on salivary cortisol at awakening and CAR in compliant seven subjects (per protocol base) (Mean ± SD)

Measurement	Unit	0w	4w	8w	
Weekday	at awakening	nmol/l	8.9 ± 4.3	11.4 ± 5.4	10.4 ± 5.8
	30 min after awakening	nmol/l	14.7 ± 7.2 #	13.7 ± 7.0	16.7 ± 8.3 #
	CAR	nmol/l	5.8 ± 4.7	2.3 ± 8.7	6.3 ± 4.8
Weekend	at awakening	nmol/l	11.5 ± 4.7	9.9 ± 5.0	10.6 ± 6.6
	30 min after awakening	nmol/l	17.4 ± 9.1 #	11.9 ± 7.9	16.9 ± 8.9 #
	CAR	nmol/l	5.9 ± 5.0	1.9 ± 5.8	6.3 ± 4.6

Significant difference between salivary cortisol at awakening and salivary cortisol 30 min after awakening (paired t-test) #: $p < 0.05$.

Table S3. Raw data of Figure 4

Subject No.	Weekday CAR (nmol/l)			PSQI-J global score		
	0w	8w	ΔCAR	ΔPSQI-J global score		
				0w	8w	
1	0.2	8.1	7.9	7	3	-4
2	13.1	4.8	-8.3	9	8	-1
3	5.0	8.1	3.1	11	4	-7
4	8.2	1.0	-7.2	10	9	-1
5	9.6	14.6	5.0	13	4	-9
6	1.5	6.9	5.4	9	8	-1
7	2.8	0.7	-2.1	10	7	-3