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Putative Role of Riboflavin in Disease Prevention

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In the early part of the twentieth century, pioneering studies on the deficiency state of pellagra in experimental animals showed that water-soluble tissue extracts could be effective in treating diseases. Further studies showed that one part of the heat-stable fraction from the mentioned extract, called yellow growth factor, had fluorescent properties. This was later purified and named riboflavin. Until 1932, when the landmark discovery of the ''yellow enzyme'' containing an isoalloxazine ring and a phosphate group was made, the physiological role of the yellow growth factor remained obscure. The synthesis of riboflavin, accomplished in 1935, was followed by the identification of the two active coenzyme forms, flavin mononucleotide (FMN) in 1937 and the clarification of the structure of flavin adenine dinucleotide in 1938, this formed from FMN.
As a water-soluble vitamin, riboflavin plays a part in a variety of oxidation-reduction reactions. Flavin mononucleotide and flavin dinucleotide act as active coenzyme forms of riboflavin that participate in a variety of reactions in the human body. Riboflavin has an important role in the fat metabolism disturbances. Through deficiency and supplementation studies and effects on the structure and function of the small intestine, riboflavin has a role in iron handling. Riboflavin is associated with compromised oxidant defense. Flavin adenine dinucleotide acts as the co-factor for 5,10 Methylenetetrahydrofolate reductase, an important enzyme, which participates in the remethylation pathway for homocysteine metabolism. Homocysteine is located at a critical metabolic crossroad and therefore both pathways, remethylation and transsulfuration; and directly and indirectly impacts all methyl and sulphur group metabolism occurring in the body. Poor vitamin status could promote higher homocysteine levels. In addition, high levels of homocysteine could be considered conditional risk factors for cardiovascular diseases. Riboflavin has also been ascribed a role in the protection against certain cancers and cataracts.
Key-words: Riboflavin, homocysteine, iron handling

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INTRODUCTION

Through its cofactor role in numerous reactions in the human body, riboflavin has been implicated as protective against diseases or conditions as diverse as cataract, oesophageal cancer, and cardiovascular disease.

Evidence comes from animal studies, epidemiological studies in humans, and a few experimental intervention studies in humans. Mechanisms for an apparent role for riboflavin as protection against certain diseases are not always understood.

Riboflavin, structure and active forms

Riboflavin, vitamin B_2, is a water-soluble vitamin, a yellow, fluorescent compound, defined chemically as 7,8-dimethyl-10-(1'-D-ribityl) isoalloxazine. The planar isoalloxazine ring provides the basic structure not only for vitamin B2 but also for the naturally occurring phosphorylated coenzymes that are derived from riboflavin. The primary form of the vitamin is as an integral component of the coenzymes flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) (1-4).

Flavin mononucleotide and flavin-adenine dinucleotide linked covalently to specific tissue proteins, generally at the 8-α methyl position of the isoalloxazine ring (5).

Dietary sources

Abundant in omnivorous diets, the Recommended Nutrient Intake (RNI) for riboflavin per day are:

Dietary reference Values for Riboflavin (mg/day) (6)

0-6 months	7-10 months	1-3 years	4-8 years	Males 9-13 years	Females 9-13 years	Males 14+ years	Females 14-18 years	Females 19 + years	Pregnancy	Lactation
0,3	0,4	0,5	0,6	0,9	0,9	1,3	1,0	1,1	1,4	1,6

The richest sources are yeast extract and offal products especially those based on liver (7). Beer is a good source as are milk, cheese and eggs. Nuts, avocados, green and yellow vegetables contain reasonable amounts; important for vegetarians who need a varied diet, in order to avoid deficiency (8). Diets based on rice are normally riboflavin deficient.

As a heat-stable vitamin, riboflavin losses in cooking are small, but it is easily destroyed by sunlight when in liquid form.

Since dietary sources of riboflavin are largely in the form of coenzyme derivatives, these must be hydrolysed prior to absorption.

Most of the vitamin B_2 in foods occurs as riboflavin, flavin mononucleotide or flavin-adenine dinucleotide, all protein bound. In the Western diet dairy products, meats, poultry, fish are good sources. Grain products, contain relatively low levels of riboflavin; however, when enriched or fortified grains, cereals, and bakery products supply larger amounts. Broccoli, turnip greens, asparagus, and spinach are also good sources.

Bioavailability of riboflavin

Dietary sources of riboflavin are largely in the form of coenzyme derivatives, mainly flavin-adenine dinucleotide and, to a lesser degree, flavin mononucleotide all protein bound. These must be hydrolysed prior to absorption, in the acidic conditions of the stomach, from pyrophosphatase to phosphatase, and then absorbed as riboflavin in the small intestine by a sodium-dependent and saturable process, within the enterocyte, and phosphorylated to flavin mononucleotide. A reasonable estimation of bioavailability is approximately 95 percent of food flavin, up to a maximum of about 27 mg absorbed per single dose (9).

The composition of the diet appears to influence the riboflavin requirements. A lower ratio of fat to carbohydrate decreased the requirement10 . This relationship was examined in elderly people.

Perhaps no more than 7 percent of food flavin is found as covalently attached 8α-flavin-adenine dinucleotide, bound to heterocyclic atoms of proteins that function catalytically. Although some portion of the 8α-(aminoacid) riboflavins are released by proteolysis they do not have vitaminic activity (11).

Riboflavin is found inside cells, in the matrix, inner and outer mitochondrial membrane, liver and kidney microsomes, predominantly as flavin-adenine dinucleotide, enzyme covalently bound (12).

Conserved through an enterohepatic cycle, riboflavin is excreted in the urine by active tubular secretion.

Deficiency symptoms

Riboflavin deficiency has been documented in developed and developing countries and across various demographic groups (13,14). The signs of ariboflavinosis are sore throat, hyperemia and edema of the pharyngeal and oral mucous membranes; cheiliosis, angular stomatitis and in a later stage glossitis, seborrheic dermatitis, photophobia and corneal vascularization, anaemia and brain dysfunction. Deficiency is most often accompanied by other nutrient deficiencies and may impair the metabolism of vitamin B_6 by limitating the amount of FMN required by pyridoxine (pyridoxamine) 5-phosphate oxidase and the conversion of tryptophan to functional forms of niacin (15). Diabetes mellitus (16,17), cancer (18) and cardiac diseases (19) are known to exacerbate riboflavin deficiency.

Assessment of riboflavin status

For assessment of riboflavin and its derivatives, a variety of available methods exist. These include Fluorometric procedures based on inherent fluorescent properties of flavins (20), competitive protein binding (21) and binding to specific apoenzymes such as D-amino acid oxidase.

Currently, high-pressure liquid chromatography (HPLC) is the method most widely employed for determination of flavins in biological fluids and tissues (22). After mild hydrolysis to convert flavin-adenine dinucleotide to the more stable flavin mononucleotide, high-pressure liquid chromatography leads to a more exact determination of flavin mononucleotide plus traces of riboflavin; and could be considered a useful indicator that reflects the functional cellularly trapped forms of riboflavin.

Riboflavin nutrition status could be generally evaluated, in both individuals and specific groups, by determining its urinary excretion and the erythrocyte gluthatione reductase activity coefficient (EGRAC) (23). Urinary riboflavin can be measured by fluorometric high-pressure liquid chromatography methods (24,25) as well as by microbiological procedures.

High-pressure liquid chromatography techniques permit easier separation of other fluorescent flavin catabolites such as 7- and 8- hydroxymethylriboflavins (26) from riboflavin and were found useful in relating the recent dietary intake to urinary output. Urinary riboflavin determinations are directly affected by several factors such as renal excretion (27), physical activity, elevated body temperature, treatment with certain drugs, and other stress conditions associated with negative nitrogen balance (28). Urinary riboflavin has been shown to increase with the administration of certain psychotropic drugs such as phenothiazine (29) antibiotics and under conditions causing negative nitrogen balance.

A decrease in urinary riboflavin excretion with an increase in physical activity (30,31) was demonstrated in some studies.

Some dietary catabolites, like 10-formylmethyl and 2'-hydroxyethyl-flavins can interfere with the response of bacteria used for assay of urinary flavin.

For adults, a low urinary concentration of riboflavin is considered to be 19 to 27 μg/g creatinine and a deficient concentration to be below 19 μg creatinine.

Erythrocyte gluthatione reductase activity coefficient is based on the degree of saturation of the apoenzyme with its coenzyme flavin-adenine dinucleotide that reflects the body stores of flavin-adenine dinucleotide. Results are expressed as an activity coefficient which is the ratio of activities in the presence of added flavin-adenine dinucleotide and without its addition, and suggested guidelines to interpreting those are as follows: 1.2 or less indicates adequate riboflavin status, 1.2-1.4 borderline-to-show status, and greater than 1.4 a clear riboflavin deficiency (32). However an upper limit of normality has been establish at 1.34 based on the mean plus 2 standard deviations of the Erythrocyte gluthatione reductase activity coefficient value of healthy elderly individuals aged 60 years and older (33). A number of physiological variables could influence the results of this determination. Erythrocyte gluthatione reductase activity coefficient is not a valid test for individuals with glucose 6-phosphate dehydrogenase deficiency, because glutathione reductase in the erythrocytes has increase avidity for flavin-adenine dinucleotide (34).

In both hypothyroidism and hyperthyroidism glutathion reductase activity is affected promoting coefficient variations (35).

Functions of riboflavin

The major function of riboflavin is to be useful as the precursor of flavin-adenine dinucleotide and flavin mononucleotide, and of covalently bound flavins. Riboflavin is primarily involved in energy-yielding metabolism. Its function is that of an electron carrier in the oxidation and reduction reactions of the flavin coenzymes. The redox functions of flavin coenzymes include both one-electron transfers and two-electron transfers from substrate to the flavin coenzyme (36). These enzymes play a fundamental role in the mitochondrial electron transport chain.

Some reactions involve transfer of a single hydrogen to a flavin, forming flavin-H˚; which is then recycled in a separate reaction. Sometimes two molecules of flavin each accept one hydrogen atom from the substrate to be oxidized. Other reactions involve the sequential transfer of two hydrogens onto the flavin, forming first the flavin-H˚ radical, then fully reduced flavin-H_2. The reoxidation of reduced flavins in enzymes that react with oxygen is a major source of potentially damaging oxygen radicals (37).

Cofactor in Macronutrient Metabolism

Flavin adenine dinucleotide (FAD) is one of the molecules considered ubiquitous throughout metabolism as a carrier of hydrogen atoms.

Riboflavin as flavin-adenine dinucleotide, is required in an important central metabolic pathway, the citric acid cycle, which provides the link between amino acid, carbohydrate and fat metabolism.

For each mole of acetyl CoA oxidized in this pathway, there is a yield of one FADH (flavoprotein reduced), equivalent to two ADP.

Riboflavin and disturbances to fat metabolism

Four studies point to the positive effect of riboflavin on disturbances in fat metabolism. In the first, the effect of riboflavin deficiency was tested on cerebrum and cerebellum of developing rat brain. The myelin lipids, cerebrosides, and sphingomyelin, as well as phosphatidylethanolamine, were considerably reduced in proportion. It is considered that riboflavin plays some role in the metabolism of essential fatty acids in brain lipids and the pathological effect of its deficiency is similar to that of fatty acid deficiency (38). The second study showed that treatment with riboflavin abolished headaches and abnormal behaviour and normalised the plasma free carnitine, when measurement of [9,10(n)-3H] palmitate oxidation by cultured fibroblasts suggested a multiple acyl-CoA dehydrogenation disorder, in a 29 years old woman with severe hyperemesis gravidarum and atypical migraine (39). A further study, after supplementation of riboflavin, clinical improvement occurred in acyl-CoA dehydrogenase deficiency; in which infants present recurrent hypoglycemia and lipid storage myopathy and increased urinary excretion of organic acids. The final study in this group, two patients with multiple acyl coenzyme A dehydrogenase deficiency, after riboflavin supplementation, flavin adenine dinucleotide and flavin mononucleotide concentrations in muscle and isolated mitochondria, and the activity of mitochondrial flavin adenine dinucleotide pyrophosphatase were total or partly corrected to normal levels and activity (40). This highlights the importance of riboflavin in fat metabolism (41). These four papers clearly illustrate the importance of riboflavin in

fat metabolism disturbances.

ROLE IN IRON HANDLING

Riboflavin deficiency studies

It has been shown that riboflavin affects the integrity of red blood cells, haemoglobin and reduced glutathione (42). Because of its oxidation-reduction potential, mainly due to nucleotides flavin-adenine dinucleotide and flavin mononucleotide, riboflavin plays an important role in the metabolism of iron, riboflavin-responsive anaemia in man having been shown, in early studies to have such characteristics features as an erythroid hypoplasia and reticulocytopenia (43). Studies on animals with severe riboflavin deficiency suggest that body handling of iron appears altered during deficiencies states (44).

Thus, it seemed highly probable that the mobilization of the second major body source for iron reduction, ferritin, would be affected. In fact observation of differences in ferritin iron accumulation between control and riboflavin-deficient rats, with controlled inanition, overcamed some potencial objections to the earlier investigations on reduced absorption or transport of iron (45-47).

Supplementation studies

The inclusion of riboflavin in a supplementation study undertaken in rural Gambia of men and children, with microcytic anaemia, improved recovery, particularly in those individuals with unusually low levels of haemoglobin at the beginning (48). Following study on rats showed that depleted ferritin stores and increased demand for iron turnover in rapid growth and pregnancy may impair iron mobilisation (49). In a subsequent study, lactating women receiving iron and riboflavin supplementation a significant increase, relative to placebo, was shown in circulating plasma iron and in iron stores (50). A further study on children with subclinicae vitamin deficiencies supplemented with multivitamin and iron, also showed marked improvements in riboflavin status, and prevented deterioration on running performance (51).

Riboflavin and iron absorption and excretion

Beyond these activities and based on the marked effects on the normal structural development of gastrointestinal tract it was suggested that riboflavin plays a part in the absorption of iron and in excretion (52). In the growing rat, liver ferritin-Fe concentrations were significantly lower in riboflavin-deficient rats after three weeks on the respective diets and remained lower until the conclusion of the experiment (53).

Riboflavin deficiency is associated with reduced in vitro iron-mobilization activity at the gastro-intestinal mucosa. Impact on iron mobilization from body stores and iron absorption may depend upon a number of factors, such as existing hepatic iron stores and the demand for rapid iron turnover (54). Early studies in the rural community in the Gambia reported anaemia in certain sections of the population, and riboflavin deficiency appears to impose some limitations on the absorption and utilization of iron (55). In a further study iron Fe absorption measured in the rat by monitoring whole-body retention of a dose of 59Fe using a small-animal gamma-counter, showed that riboflavin deficiency could be associated with a reduction in the percentage of the dose absorbed and an increase in the rate of loss of iron post absorption (56). In a study of riboflavin deficiency and iron absorption in adult Gambian men the results indicate that the efficacy of iron utilization is impaired but that iron absorption is unaffected (57).

THE EFFECT OF RIBOFLAVIN ON THE STRUCTURE AND FUNCTION OF THE SMALL INTESTINE

Riboflavin deficiency is thought to be one of the factors, which elicit iron loss from the body because in gastrointestinal mucosa, one of the major absorption sites, the flavin-dependent iron mobilization from ferritin, an increased rate of turnover of epithelial cells will increase this loss. Another effect of riboflavin deficiency on gastrointestinal Fe distribution and loss was studied in weaning rats; the reported enhanced iron loss was due, predominantly, to an accelerated rate of small-intestinal epithelial turnover (58). Depletion of riboflavin was associated with increased villus length and a proportional increase in the number of cell positions along villi in rats, and did not influence the number of mucus-producing goblet cells or the amount of mucosal glycoprotein in the small intestine (59).

The effects on the development of the gastrointestinal tract

The cytokinetics and structure of the small intestine may be altered in weaning rats in riboflavin deficiency induced at weaning and this impairs the normal increase in villus number. Prolonged deficiency leads to an adaptative increase in length of villi and depth of crypts (60). Reversibility was studied, also in weaning rats, and the results show that the small intestine cannot readily recover from a period of riboflavin deficiency induced at weaning. These results highlight the critical nature of the weaning period for gastrointestinal development and the importance of adequate nutrition during infancy (61). The earliest point at which riboflavin deficiency affects post-weaning bowel development in rats, was studied and the results observed that developmental changes to the duodenal crypt arise shortly after circulating riboflavin measurements show evidence of deficiency (62).

Participating in oxygen transport and oxidative phos-

phorylation, iron limits the synthesis of haem and controls the activity of the electron transport chain. Anaemia reduces work performance in humans and produces an effect on the efficiency of the electron transport chain in generating energy through oxidative phosphorylation.

Antioxidant activity

Often neglected as an important dietary antioxidant, riboflavin in its role as a precursor to flavin mononucleotide and flavin-adenine dinucleotide is extremely powerful. The Glutathione redox cycle provides a protective action against the scavenging of lipid peroxides (63).

Glutathione reductase requires flavin-adenine dinucleotide. A diminished conversion of oxidized glutathione occurs as a result of decreased activity of glutathione reductase (64), which leads to diminished concentrations of the substract for glutathione peroxidase and S-transferase, limiting the rate of degradation of lipid peroxides and xenobiotic materials (65). NADPH provides reducing equivalents, another substract required by glutathione reductase, primarily generated by glucose-6-phosphate dehydrogenase, the activity of which is significantly diminished during riboflavin deficiency. Riboflavin deficiency as widely reported, is associated with compromised oxidant defense. The use of riboflavin, on the other end, as a reducing agent for 48 hours, decreased the parasite methemoglobin level, food vacuole size and inhibited asexual parasite growth in cultures of Plasmodium falciparum (66). Combination of riboflavin with mefloquine, pyrimethamine, and quinine showed a marked potentiation of the actives of these drugs against asexual-stage parasites in vitro (67).

Reduced glutathione (GSH) is one of the most important endogenous antioxidants in the cell. Its synthesis is dependent on the activity of γ-glutamylcysteine synthase, and the availability of the substract, cysteine, which is either derived from the diet or protein catabolism, or synthesized from methionine in the liver by the transsulfuration pathway. An increasing supply of cysteine for Reduced glutathione synthesis is produced when homocysteine transsulfuration is favoured over remethylation.

The role of riboflavin in homocysteine metabolism

The possible role of homocysteine, a thiol-containing amino acid which is naturally found in the body as an intermediary product in methionine metabolism in the pathogenesis of vascular disease, is at present an important subject of study (68).

Transsulfuration pathway for homocysteine metabolism

In this pathway, methionine is sequentially converted into cysteine via several enzymatic steps, the first step it is catalysed by methionine adenosyltransferase, and ATP-dependent activation, to S-adenosylmethionine. Subsequent demethylation and removal of the adenosyl moiety yields homocysteine. To form cystathionine, homocysteine condenses with serine in a reaction catalysed by cystathionine synthase and dependent on pyridoxal 5-phosphate (the active form of vitamin B6). Free cysteine is released after cleavage of cystathionine, catalysed by cystathionine γ–lyase.

Methionine and homocysteine are readily interconvertible, but the subsequent step, the formation of cystathionine, is irreversible. The regulating activity of remethylation and the transsulfuration pathway appears to depend on the availability of methionine. It is on homocysteine levels that the main regulatory control appears to be exerted. Homocysteine is remethylated by methionine synthase or betaine-homocysteine methyltransferase to yield methionine, when needed. Its metabolism is accelerated via the cystathionine β-synthase reaction when methionine is in excess (69).

Serum homocysteine concentrations are sensitive to blood levels of folate. Flavin-adenine dinucleotide acts as the co-factor for 5,10 Methylenetetrahydrofolate reductase, an important enzyme, which participates in the remethylation pathway for homocysteine metabolism. Recognized for its importance as coenzyme of cystathionine β-synthase and cystathioninase, vitamin B6 participates in the inactivation of homocysteine.

Remethylation pathway of homocysteine metabolism

In a vitamin B_{12}-dependent reaction, a methyl group is donated to homocysteine by 5-Methyltetrahydrofolate; or by betaine, to regenerate methionine. Homocysteine catalise the reaction, with methylcobalamin as coenzyme.

Folate and vitamin B_{12}, are both, independent in the methyl donation of betaine. Adequate riboflavin nutrition is required for efficient utilization of dietary folic acid.

The intermediate metabolite homocysteine is located at a critical metabolic crossroad and therefore both pathways, directly and indirectly impacts all methyl and sulphur group metabolism occurring in the body.

Riboflavin is considered, among others, as a determinant of plasma total homocysteine concentration in the Framingham Offspring cohort (70).

Evidence for a role for riboflavin as protective against diseases

Literature regarding evidence for a role for riboflavin, as protective against diseases is not so crowded, and landmark studies may be not difficult to recognize. Trials are generally concise and include important information that is provided in adequate detail for careful evaluation. But, some data are of interest to some parts of the scientific community and not to others. Recent analysis deserves public dissemination and it is believed that an

enormous amount of useful knowledge can be derived from carefully conducted studies. Full dissemination of obtained results is likely to be helpful to the scientific community.

RIBOFLAVIN AND CATARACTS

Cataract pathophysiology

Detoxification of xenobiotics is one of the major functions of reduced glutathione. High concentrations of reduced glutathione protect against oxidation and cross-linking the protein-bound Sulfhydryl-groups of crystallins, the transparent tissues of the eye, which have a relatively xenobiotic metabolism. Ageing generally reduces the lens metabolic efficiency, thus increasing its susceptibility to noxious factors; and could also promote the interactions for cataract noxae to induce the formation of a variety of cataract, associated with high protein-related light scattering and discoloration.

Vitamins with antioxidant properties have been implicated in the development and progression of cataract. In numerous studies riboflavin was found to have benefits arising from utilization.

Epidemiology of cataract

Riboflavin deficiency was first postulated to be involved in the formation of cataract by Day, Langston & O'Brien (71) in 1931. Since then, there have been several human studies into the effects of riboflavin deficiency, and it is now widely agreed that riboflavin deficiency appears to be a significant predisposing factor for cataract (72). Studies in animals and also epidemiological and interventional studies in humans have revealed the important role of riboflavin (73).

In the Lens Opacities Case-Control Study, which assessed the risk factors for various types of cataract among participants aged 40 to 79 years, the risk factors for age-related cataracts was evaluated. The results support a role for nutrition in cataractogenesis (74). Lens opacities were associated with lower levels of riboflavin, and are compatible with the dietary intake and medical history results of Lens Opacities Case-Control Study (75). It was found an increased risk with low levels of several nutrients including riboflavin. This kind of study could be considered an efficient design for the evaluation of potential factors for cataract.

But unlike the cohort study, with few exceptions a case-control study cannot demonstrate the risk of developing the disease in individuals with a suspected risk factor.

The Blue Mountains Eye Study (76), a cross-sectional study, where 79% of the participants aged 49 to 97 years attending, reports on the relationship between the three principal types of cataract, nuclear, cortical and posterior subcapsular, and a wide range of dietary macronutrients and micronutrients, including riboflavin, a very important element supporting the above mentioned hypothesis. Results showed a statistically significant dose trend for riboflavin supplements on cortical cataract; riboflavin (odds ratio 0.8, confidence interval 0.6 to 1.0, P=.05) and niacin (odds ratio 0.7, confidence interval 0.1 to 1.0, P=.04) both shown to exert a weaker protective influence. The cross-sectional nature of this study, the selection bias, confounding and multiple comparisons could be considered as limitations for out coming results.

The low statistical power may also have contributed to random error, because many subgroups had relatively small number of supplement users.

The full assessment of the temporal relationship between vitamin supplementation use and cataract was not permitted due to the nature of the study, which is important in such analysis. The dietary vitamin intake was not corrected, which could contribute also for confounding the findings.

Riboflavin appears to be protective in isolation or as constituent of multivitamins preparations.

The Linxian Cataract Study (77), a large intervention trial, was probably the first randomised population trial in the world; suggesting a general benefit and support the general notion that riboflavin supplementation were beneficial. Examinations at the end of the intervention showed a reduction of 36% in the prevalence of nuclear cataract for individuals aged 65-74 years, who received riboflavin plus niacin compared to those who did not. Although exists some inconsistencies with the results from other clinical trials like The Physician's Health Study, The Lixian Study provide a hopeful sign that riboflavin supplementation may lower the risk of nuclear cataract, and suggest lines of further research to confirm the protective effect of riboflavin (78).

Riboflavin and cardiovascular disease

Cardiovascular diseases (CVD) remain a major cause of death and morbidity in developed countries. Since the aetiology of cardiovascular disease is influenced by environmental and genetic factors (79), studies at cellular, molecular and genetic levels are important to understand cardiac dysfunction. Advances in understanding vascular smooth-muscle and endothelial cells are also important; endothelial cells are quite active metabolically and normally produce substances that affect the vascular lumen.

Flavin-adenine dinucleotide could prevent the decrease in ventricular multiple response threshold and the disturbance of mitochondrial function (80).Riboflavin supplementation has cardioprotective effects in cardiac reoxygenation damage and these effects are mediated by flavin reductase (81).

Vitamin B2 could be considered an active supplement to the antioxidant and vitamin status of patients with hypertension and ischemic heart disease (82). Riboflavin is one of B-vitamins on which the remethylation pathway of homocysteine is dependent; as is the trans-sulfuration

pathway. Vitamin B2 is required for intracellular homocysteine metabolism (83). To date, even though riboflavin plays a crucial role in both the trans-sulfuration and remethylation pathways of homocystein metabolism, the relationship between riboflavin status and homocysteine levels has been investigated in few studies.

Mechanisms of damage

Some reactive oxygen species (ROS), such as hydrogen peroxide, H_2O_2, produced mainly in mitochondria, after oxygen reduction to water in the electron transport chain (84), react with divalent cations in the Fenton reaction, thereby forming the very reactive hydroxyl radical ($OH^{\cdot}$).

Reactive oxygen species produced through the metabolism of homocysteine, could promote endothelial damage (85), but the actual mode is still unclear. Homocysteine facilitates the generation of hydrogen peroxide (86). By creating oxidative damage to LDL cholesterol and endothelial cell membranes, hydrogen peroxide can catalyse injury to vascular endothelium (87). The platelet aggregation induced by hydrogen peroxide could be inhibited by flavin adenine dinucleotide via the glutathione reductase and peroxidase system (88).

The isolated rat mesenteric arterial bed was used to examine the activity of flavin-adenine dinucleotide, in the study of pivotal role of phosphate chain length in vasoconstrictor actions of adenine dinucleotides in rat mesenteric arteries, and showed those three or less phosphates are vasodilators (89). By reacting with homocysteine, nitric oxide released by endothelial cells protects them from damage, forming S-nitrohomocysteine, which inhibits hydrogen peroxide formation. This protective mechanism can became overloaded, as homocysteine levels increase, allowing damage to endothelial cell to occur (90). Atherosclerotic plaque formation could result from the combination of oxidative damage and endothelial collagen promoted by high levels of homocysteine.

Hyperhomocysteinaemia

High levels of homocysteine could be considered conditional risk factors for cardiovascular diseases (91). Homocysteine-related abnormalities are also thought to contribute to birth defects and dementia, and there are many common acquired diseases, drugs and genetic disorders which adversely affect the metabolism of homocysteine. The major circulating forms of homocysteine are protein bound, with twenty per cent unbound.

Elevated levels on plasma of total homocysteine in concentrations greater than 16 (mol/l, result from certain drugs utilisation such as folate, B6 and B12 antagonists. Another factor to be considered could be age increase. A thermolabile polymorphism of MTHFR is a more recent approach to high plasma homocysteine levels investigation, with a range in homozygous state varying between 5 to 15 per cent, depending on populations, and where heterozygotes are meaningful (92). Studies among coronary artery disease and premature vascular disease suggest that this genetic defect could contribute to development of cardiovascular diseases.

Maintained by enzymes, the methionine metabolism could promote high levels of Hcy if folate, vitamine B_6, vitamine B_{12} are not deficient. Adequate riboflavin nutrition is required for efficient utilization of dietary folate (93).

While the role of reactive oxygen species in the vasculature is far from clear, evidence suggests that homocysteine is a contributing source of reactive oxygen species in endothelial cells, vascular smooth muscle cells and intact aortas (94).

The importance of riboflavin in homocysteine metabolism is recognized. The first study, in humans, showing the above mentioned relationship was Determinants of Plasma Concentration in The Framingham Offspring cohort. In this cohort was observed an association between plasma tHcy and nutritional factors where riboflavin was included. The geometric mean tHcy was 23% higher in persons aged ≥ 65 years and 11% higher in men. Also the use of vitamin B supplements was associated with significantly lower tHcy concentrations (95).

In order to examine the association of a single tHcy measurement on subsequent hospitalisations due to cardiovascular diseases, a population-based prospective cohort study was conducted in Norway; also known as The Hordaland Homocysteine Study. Risk of cardiovascular diseases hospitalisations increased significantly with increasing baseline tHcy only in the oldest aged group, 65 to 67 years at baseline. Plasma tHcy levels is a strong predictor of cardiovascular diseases hospitalisations only in elderly individuals, and especially among those pre-existing cardiovascular diseases (96).

Association between B vitamin and plasma homocysteine concentration in the general Dutch population aged 20-65 y (97) was the first study in which the association between dietary intake of riboflavin was investigated for a large population-based sample. The population of this study consisted of a random sample of subjects aged 20-65 years from 3 cities in the Netherlands. Riboflavin intakes were calculated by using an extended version of the 1996 computerized Dutch food-composition table. Folate data were derived from a validated high-pressure liquid chromatography method with which Dutch foods were analysed. Nonfasting venous blood samples were used.

Folate was the most important dietary determinant of the plasma tHcy, and high dietary folate can make a substantial contribution to a reduction in plasma tHcy in the general population, which is important because each 1μmol/L decrease in tHcy may be associated with a 10% reduction risk of cardiovascular disease. No association between plasma tHcy and riboflavin was observed. As a cross-sectional study was limited the identification of causal relations. In previous studies, as above mentioned, subjects were older.

More recently, the effect of riboflavin supplementation

on plasma homocysteine in elderly people with low riboflavin status was studied, in a double blind, randomised, placebo-controlled riboflavin supplementation trial. McKinley et al (98) found that despite the metabolic dependency of tHcy on riboflavin, it did not prove to be an effective homocysteine-lowering agent, even in the face of sub-optimal riboflavin status. A supplementation level of 1.6 mg/day riboflavin for 12 weeks was chosen based on a previous riboflavin intervention (99). Even when the response to intervention was examined separately in those subjects deemed to have inadequate biochemical status of either nutrient at baseline, supplementation with riboflavin resulted in a significant response in the status of whichever nutrient was low, riboflavin or vitamin B6. But the small number of subjects classified as having low status for both vitamins (n=7), riboflavin supplementation appeared to improve the status of each vitamin, but no firm conclusions can be drawn from such a small sample.

Low dose of riboflavin was also used in order to make the results more relevant for prevention of hyperhomocysteinemia through modification of dietary intakes, via fortification of foods.

Fortification of foods can be a useful tool in combating micronutrient deficiencies, and successful application of food fortification technology is based largely on compatibility of vehicle, fortification and process. Riboflavin is unstable in alkaline medium; it is very sensitive to light, particularly in presence of ascorbic acid.

The requirement regarding riboflavin should be considered looking for a bigger amount used for fortification and probably a homocysteine lowering effect will be effective.

Evidence that homocysteine is an independent risk factor for vascular disease is growing, but is important to understand the determinants of plasma homocysteine. Low folate status could be considered the most important determinant of mild-to-moderate hyperhomocysteinemia. It is well known that the folic acid fortification of enriched grain products in the United States dramatically altered the prevalence of elevated plasma homocysteine concentrations associated with low folate. Other determinants, like riboflavin, could assume greater importance and efforts to identify additional risk factors for mild-to-moderate hyperhomocysteinemia needs to continue.

Riboflavin and esophageal cancer

Recent studies confirm that nitrosamines, well known carcinogens, may increase carcinogenesis due to dietary riboflavin deficiency (100). Damage of DNA from its coenzyme action with enzymes of cytochrome P450 could be avoided by riboflavin protection (101).

In the aetiology of oesophageal cancer, a nutritional imbalance could be considered an important factor. To test this hypothesis, two large intervention trials using vitamins and minerals supplementation, were conducted in a population with the highest rate of esophageal cancer in the world, in Linxian, China (1985-1991).

From the General Population Trial, results showed that riboflavin plus niacin decreased the incidence of oesophageal cancer by 14 per cent approaching significance.

In the Dysplasia Trial results showed that in the supplementation group compared to the placebo group the risk of oesophageal cancer mortality is 16 per cent lower and oesophageal/gastric cardia cancer mortality 8 per cent lower. The suggestion of benefit from vitamin and mineral use should be observed in a longer follow up; although the results were not statistically significant.

The measurement of cell proliferation with the utilization of tritiated thymidine labelling evaluated in 512 endoscoped patients showed that the participants who were supplemented were significantly more likely to revert to non-dysplastic cytology at both 30 and 72 months.

The results can be considered very important because this was the first randomised trial in the world where a positive correlation between nutritional supplementation and reduction of incidence from human cancer was shown.

CONCLUSIONS

Riboflavin has an important role as cofactor in metabolism, participates in the antioxidant activity and in iron metabolism.

Has a recognized importance as a determinant of plasma total homocysteine, and evidences for a role for riboflavin as protective against diseases appear from studies in humans.

A hopefull sign that riboflavin supplementation may lower the risk of nuclear cataract comes form the Lixian study. Through a randomised trial, a positive correlation between riboflavin and oesophageal cancer was suggested.

Few studies estimating the riboflavin requirements on the elderly, have been conducted to date.

Not only do they form an increasingly large proportion of the population, but also constitute the population group that consumes the largest number of prescribed and over-the-counter medications. This could interfere with riboflavin utilization by impairment of the conversion from vitamin through its coenzymes.

Malabsorption and poor diet, which the elderly are more prone to, may interfere also, and may be intensified if alcohol abuse exists.

Dietary habits need to be altered in order to improve vitamin supply, particularly in elderly. A strong case can be made for preventing the marginal or manifest riboflavin deficiency states that may contribute substantially to this potentially important risk factor for cardiovascular disease, the largest cause of mortality among elderly.

Persons undergoing haemodialysis or peritoneal dialysis are likely to require extra riboflavin along with other B vitamins.

The efficacy of riboflavin supplementation for the prevention and treatment of anaemia must be further

evaluated since there is strong evidence that riboflavin deficiencies affect iron utilization from supplements and are important on a large scale.

No cases of toxicity from ingestion of riboflavin have been reported, since the capacity of the normal human gastrointestinal tract to absorb this soluble vitamin is rather limited.

Priority should be given to studies for setting Estimated Average Requirements for riboflavin for the elderly, defining values for clinical adequacy and inadequacy.

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