Cellular and clinical implications of glutathione

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Glutathione (GSH), a tripeptide consisting of L-γ-glutamyl-L-cysteinylglycine (Fig. 1), is the most abundant intracellular thiol compound present in virtually all mammalian tissues. Functions of GSH in reductive processes are essential for the synthesis and also degradation of proteins, formation of the deoxyribonucleotid precursors of deoxyribonucleic acid (DNA), regulation of enzymes, and protection of the cells against reactive oxygen species and free radicals produced even in normal metabolism. By its multifunctional properties GSH attracts the interest of researchers in various subjects such as enzyme mechanisms, biosynthesis of macromolecules, intermediary metabolism, drug metabolism, radiation, cancer, oxygen toxicity, transport, immunology, endocrinology, environmental toxins, aging and exercise. Most of the new information about GSH biochemistry is produced with selective inhibitors of GSH turnover. Selective modulation of GSH metabolism also makes new therapeutic approaches possible.

Glutathione metabolism still looks promising to scientists in cell biology and exercise physiology thanks to its central role in the antioxidant defense and modulation by specific inhibitors for experimental purposes. In addition, glutathione may have clinical importance since enzyme deficiencies of the glutathione metabolism may cause haemolytic anaemia and neurologic symptoms in children and decreased glutathione levels have been reported in several diseases including acquired immune deficiency syndrome (AIDS), diabetes, adult respiratory distress syndrome, and Parkinson’s disease. Therefore, metabolism and functions of GSH, the manipulating strategies of GSH level in vitro and in vivo, in this regard effects of physical exercise on tissue GSH levels, and cellular and clinical implications of GSH have been reviewed under the light of the recent evidence.

Metabolism of glutathione

γ-Glutamyl cycle, a series of six enzyme-catalyzed reactions which account for the synthesis and degradation of GSH, has been formulated by Alton Meister. GSH is synthesized intracellularly by the consecutive actions of γ-glutamylcysteine synthetase (1) and GSH synthetase (2) (Fig. 2). γ-Glutamylcysteine synthetase is feedback inhibited by GSH. The breakdown of GSH and also of glutathione disulfide (GSSG) and S-substituted GSH is catalyzed by γ-glutamyl transpeptidase (3), which catalyzes transfer of the γ-glutamyl moiety to acceptors - amino acids, e.g. cystine, glutamine, and methionine, certain dipeptides, water, and GSH itself. GSH occurs mainly intracellularly, and a major fraction of the transpeptidase is on the external surface of the cell membranes. GSH transported across cell membranes interacts with γ-glutamyl transpeptidase. γ-Glutamyl amino acids formed by γ-glutamyl transpeptidase are transported into cells. Two enzymes of the cycle also function in the metabolism of S-substituted GSH derivatives, which may be formed nonenzymatically by reaction of GSH with certain electrophilic compounds or by GSH S-transferases (7). The γ-glutamyl moiety of such conjugates is removed by the action of glutamyl transpeptidase (3), a reaction facilitated by γ-glutamyl amino acid formation. The resulting S-substituted cysteinylglycines are cleaved by dipeptidase (6a) to

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yield the corresponding S-substituted cysteines, which may undergo N-acetylation (8) or an additional transpeptidation reaction (3a) to form the corresponding γ-glutamyl derivative. These mercapturates are water soluble and can be excreted in the urine. Intracellular GSH is converted to GSSG by selenium containing GSH peroxidase (9), which catalyzes the reduction of H₂O₂ and other peroxides. Selenium supplementation generally protects the cells against oxidative stress. However, even though the GSH peroxidase activity was increased 44% by selenium supplementation in Renga cells, it did not help to protect them against oxidative stress in vitro in our experiments. There is evidence that certain GSH S-transferases can also catalyze such reactions reducing peroxides. GSH is also converted to GSSG by transhydrogenation (10). Reduction of GSSG to GSH is mediated by the widely distributed enzyme GSSG reductase (11) which uses nicotinamide adenine dinucleotide phosphate (NADPH). Extracellular conversion of GSH to GSSG has also been reported; the overall reaction requires O₂ and leads to formation of H₂O₂. GSSG is also formed by reaction of GSH with free radicals.

Regulation of GSH levels in vivo must be looked on in terms of the entire organism. Because, while the liver synthesizes and also exports large quantities of GSH into the blood, the kidneys take up the GSH from the plasma through breakdown of the GSH via the γ-glutamyl transpeptidase reaction. By their γ-glutamyl transpeptidase activity the lung and intestinal epithelium can also utilize the extracellular GSH.

Functions of glutathione

Glutathione with its sulfhydryl group functions in the maintenance of sulfhydryl groups of other molecules (especially proteins), as a catalyst for disulfide exchange reactions, and in the detoxification of foreign compounds, hydrogen peroxide and free radicals. A number of potentially toxic electrophilic xenobiotics (such as certain carcinogens, bromobenzene, chlorobenzene) are conjugated to the nucleophilic

![Glutathione metabolism diagram](image-url)
GSH (Fig. 2, 7). The enzymes catalyzing these reactions are called glutathione S-transferases and are present in high amounts in liver cytosol and other tissues. Glutathione-conjugates are subjected to further metabolism prior to excretion (Fig 2, 6a, 3a, 8). If the potentially toxic xenobiotics were not conjugated to GSH, they would be free to combine covalently with DNA, ribonucleic acid (RNA), or cell protein and could lead to serious cell damage. GSH is thus an important defense mechanism against toxic compounds, such as some drugs and carcinogens.

GSH has other important functions in human cells, apart from its role in xenobiotic metabolism. It participates in the decomposition of potentially toxic hydrogen peroxide in the reaction catalyzed by GSH peroxidase. GSH is an important intracellular reductant, helping to maintain essential SH groups of enzymes in their reduced states. When GSH acts as a reducing agent, its SH becomes oxidized and forms a disulfide link with other molecule of GSH (Fig. 2, 9). Oxidized glutathione (GSSG), in turn, can be reduced to GSH by the action of GSSG reductase, in a reaction using NADPH (Fig. 2, 11). NADPH is recycled by glucose-6-phosphate dehydrogenase via the pentose phosphate pathway (Fig. 2, 12), which is particularly important in red blood cells. As already mentioned, a metabolic cycle involving GSH as a carrier has been implicated in the transport of certain amino acids across membranes in the kidney. The first reaction of the cycle is:

Amino acid + GSH → γ-Glutamyl amino acid + Cysteinylglycine

This reaction helps to transfer certain amino acids across the plasma membrane, the amino acid being subsequently hydrolyzed from its complex with GSH and the GSH being resynthesized from cysteinylglycine. The enzyme catalyzing the above reaction is γ-glutamyl transpeptidase. It is present in the plasma membrane of renal tubular cells and in the endoplasmic reticulum of hepatocytes. In addition, the activities of many enzymes such as glyoxylase and formaldehyde dehydrogenase are influenced by GSH and other thiols, reflecting a physiological regulatory role of GSH.

It is postulated that ionizing radiation would rapidly oxidize the thiol groups of cells. In accordance with this hypothesis, radiation decreases cellular concentration of GSH and lead to formation of GSSG. Radiosensitivity of cells depends on the intracellular thiol level. Administration of various thiols can protect the cells and animals against the effects of radiation. The effects of radiation in air are generally greater than those found under hypoxic conditions. The oxygen effect might be explained by the fact that in O2, cells produce more reactive oxygen compounds, which are normally destroyed by reaction with GSH. Under hypoxic conditions, more GSH would be available to react with radiation induced radicals.

Intracellular thiol redox state has been observed to be a major regulator of nuclear factor kappa-B response. GSH as an endogenous thiol and thiol agents such as lipote and N-acetylcysteine have been effective in inhibiting the activity of this transcription factor induced by various cytokines, phorbol ester or oxidant. The activity of the nuclear factor kappa-B may be involved in the pathogenesis of several human diseases including AIDS, cancer, atherosclerosis and diabetic complications.

Central role of glutathione in the antioxidant network

In biological systems, oxidative stress refers to an imbalance between pro- and anti-oxidants in favor of the former, which is very harmful for the cells. Defense against oxidative stress is primarily dependent upon an orchestrated synergism between several endogenous and exogenous antioxidants. For example, vitamin E is a major lipid phase antioxidant that protects against oxidative lipid damage. In biological membranes vitamin E is present in a low molar ratio compared to the abundance of phospholipids that are highly susceptible to oxidative damage. Vitamin E is continuously recycled as it acts as an antioxidant. Exogenous nutrients such as vitamins C and E are not produced in the human body. Thiols such as GSH and dihydrolipoate support vitamin C and E recycling (Fig. 3).

Glutathione levels in biological samples can be measured by various methods. In widely used spectrophotometric GSSG recycling assay, the rate of formation of 2-nitro-5-thiobenzoic acid is measured at 412 nm in a system containing 5,5'-dithiobis(2-nitrobenzoic acid), GSSG reductase, phosphate buffer, ethylenediaminetetraacetic acid, NADPH, and sample. Total glutathione (GSH+GSSG) of the sample is determined by comparison with an appropriate standard curve. GSSG is measured by masking GSH with 2-vinylpyridine by the same procedure. GSH and GSSG have also been determined by high-performance liquid chromatography giving very similar results to the enzymatic method. Monochlorobimane-flow cytometry offers a qualitative estimate of GSH/thiol levels in cells derived from tumor and normal tissue specimens.
Strategies to manipulate glutathione levels in vitro and in vivo

Depletion of cellular GSH

Studies on the effects of GSH depletion should yield significant information about the physiological functions of this compound. Three approaches exist for depleting cell GSH level: First, GSH can be directly complexed to an electrophilic agent such as diethylmaleate (DEM), bromobenzene, or forone via the GSH transferase reaction. GSH levels are rapidly depleted by these reagents, but in most cells there is a residual pool of GSSG that is not easily depleted by these agents. High levels of DEM and other electrophilic agents tend to be toxic and may have other nonspecific effects on cell proteins. Second, depletion of GSH in cells may be achieved by inhibition of its synthesis with buthionine sulfoximine (BSO), the specific inhibitor of γ-glutamyl cysteine synthetase. The rate at which cells are depleted varies with the turnover rate of GSH in the cells, but significant depletion occurs for most cell types within a few hours, and low levels of GSH are maintained for longer periods of time. The third method for depletion of cellular GSH is to subject cells to oxidant stress, particularly in the presence of an inhibitor of GSSG reductase, such as N,N-bis(2-chloroethyl)-N-nitrosourea and adriamycin. In this case oxidized GSSG is transported out of cells, or forms mixed disulfides with cellular or extracellular proteins resulting in a net loss of GSH. Of them the excellent method for depletion of intracellular GSH is administration of sulfoximine inhibitors of γ-glutamylcysteine synthetase; BSO is a potent and selective inhibitor of GSH synthesis that is highly effective in in vitro and in vivo conditions. Human lymphoid cells depleted of GSH exhibits increased sensitivity to irradiation. GSH depletion by sulfoximine inhibitors renders tumor cells more susceptible to irradiation and to chemotherapeutic agents that are detoxified by reactions involving GSH. In vivo studies of this kind have also been carried out. It is possible to administer BSO by oral or parenteral way. When BSO is given to rats by subcutaneous injection (4 mmol/kg) the level of GSH in liver and kidney declines rapidly to about 20% of the control, with a mild (70% of control) decrease in heart and skeletal muscle and further decrease occurs much more slowly. This may reflect the existence of an intracellular pool of GSH in mitochondria that has relatively slow turnover. Sen et al. have also reported substantial decreases in the level of GSH in liver, heart, lung and skeletal muscles of the rats after intraperitoneal BSO (6 mmol/kg, for 4 days) treatment.

The effects of glutathione depletion on basic cell functions

The mere abundance of GSH suggests that severe depletion of GSH should have major consequences. Therefore it seems rather surprising that GSH depletion per se has no direct consequences in the form of acute toxicity. However, this does not imply that GSH depleted cells behave like normal cells being just more susceptible to chemical or oxidative stress. Severe depletion of GSH affects numerous cell functions: The GSH status affects the synthesis of two major cellular polymers, i.e. proteins and DNA. Oxidation or depletion of GSH may decrease protein biosynthesis. During DNA synthesis, GSH is needed as a reducing equivalent for glutaredoxin, which in
Acute GSH depletion to 5% of control level by BSO plus dimethylfumarate has a marked effect on DNA synthesis and plating efficiency in murine mammary carcinoma cells. Cultured cells become quiescent under these conditions. The antibody-dependent and cell-mediated cytotoxicity in GSH-depleted experimental approaches show that cells not only GSH dependent, e.g. glutathione synthetases, glutathione peroxidases, glutathione transferases, leukotriene C4 synthetase 3-iodinase, glutaredoxin, and glyoxylase. The activity of further enzymes may be regulated by thiol disulfide exchange, and thus depend on the GSH status. Therefore many experimental approaches show that cells not only become more susceptible to any further challenge, but their basic functions are also perturbed by the extensive GSH depletion.

Increasing cellular glutathione

Elevation of GSH in cultured cells can be achieved by a variety of methods including: enhancement of uptake of cystine; reduction of cystine to cysteine in cell culture medium; provision of alternate sources of cyst(e)ine to the cell; by pass of the γ-glutamylcysteine synthetase step of GSH regulation by providing γ-glutamylcysteine directly to the cells; and bypass of GSH synthesis completely by adding GSH or derivative directly to the cells. Cystine transport can be increased by DEM treatment or by exposures to oxygen-free radical-generating systems. Reduction of cystine to cysteine can be achieved by the addition of reducing agents such as cysteamine, N-acetylcysteine, lipoate or even GSH to the culture medium. Cysteine can be added directly to cell cultures, but it is quickly oxidized to cystine and can be toxic to some cells, possibly due to the formation of thionyl radicals that could initiate lipid peroxidation. GSH in excess has also been shown to be a source of thionyl radicals under certain conditions. Alternately, the cysteine analogue oxothiazolidine carboxylate can be added to cultures in place of cysteine. It enters cells independently of the cystine transport pathway and is converted to cysteine intracellularly by the action of oxoprolinase. In cells with high γ-glutamylcysteinyl transpeptidase levels, such as kidney and some epithelial cells, γ-glutamylcysteine may be taken up directly by the cells and used for GSH synthesis. Incubation of cells with ethyl or methyl ester of GSH is a final strategy used for increasing cellular GSH. These compounds readily cross cell membranes and are hydrolyzed to GSH intracellularly, providing a source of GSH independent of all regulatory transport and synthetic processes. However, these GSH esters have toxic effects. Thus the choice to manipulate cell GSH levels is rather extensive. Availability of these methods makes possible to investigate the contribution of GSH to cellular processes from oxidant defenses to the regulation of growth.

While GSH certainly is essential, it should not be overlooked that there is some evidence that GSH is not always to the good. Cysteinyglycine, the product of the cleavage of GSH by γ-glutamyltranspeptidase, is a potent mutagen in the Ames test. It does not directly bind to DNA, but formation of a thiocarbonyl anion gives rise to reactive oxygen species, which induce damage.

Increasing glutathione levels in vivo

Enhancing tissue GSH reserve in mammals is a challenging task particularly because GSH per se is not available to tissues when administered orally or injected intraperitoneally. N-Acetylcysteine and α-lipoic acid are the most promising drugs in this respect. N-Acetylcysteine is a proglutathione drug to increase the GSH level of the cells and tissues in vivo. Lipoate is present in natural resources as the form of lipoalysyme. Lipoyc can bypass the adverse effects of excess glutamate by reducing extracellular cystine to cysteine. Further information about them can be found in more comprehensive reviews.

Effects of physical exercise and training on tissue glutathione levels

Physical exercise and training affect tissue glutathione homeostasis. While total GSH (GSH + GSSG) level does not change, blood GSSG level increases after acute exercise in healthy and diabetic men and rats. Generally GSH depletion in various tissues including liver, heart, skeletal muscles and kidney is reported after acute exhaustive exercise in rat. These data suggest that GSH in blood and tissues is being oxidized to remove increased reactive oxygen species due to acute exercise.

On the other hand, physical training strengthens GSH dependent antioxidant defense mechanism. An increase in plasma GSH level in young rats after 5 weeks of exercise and unchanged plasma GSSG level, i.e. prevention of blood GSH oxidation, in trained athletes after the race are reported. Physical training (40 km/day for about one year) increases total...
GSH level in both liver and red gastrocnemius muscle, while immobilization (11 wk) decreases muscle GSH level in beagle dogs. The activity of γ-glutamyl transpeptidase is upregulated by training in active leg but not passive trunk muscles. Skeletal muscle GSH peroxidase and GSSG reductase and liver GSH S-transferase activities are also upregulated in these dogs by training. Increased skeletal muscle GSH level is also reported after sprint type training. These studies clearly show the beneficial effects of physical training on glutathione homeostasis.

**Clinical implications**
Tissue glutathione levels can be affected by various physiological and pathophysiological situations (Table 1). Deficiencies of specific enzymes of GSH metabolism are not common but can be seen in humans. Of them GSH synthetase and γ-glutamylcysteine synthetase deficiencies result in low levels of GSH. Glutathione synthetase deficiency can be generalised to all tissues or can only be restricted to erythrocytes. In generalised GSH synthetase deficiency, overproduction of 5-oxoproline due to lack of feedback inhibition of γ-glutamylcysteine synthetase by glutathione exceeds the capacity of 5-oxoprolinase (Fig. 2). So that a substantial amount of the 5-oxoproline is excreted in the urine. In addition, these patients show haemolytic

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*When blood samples were stored at 4°C for 4 days either as whole blood or as washed erythrocytes with or without glucose.
†After 20 hr incubation of lymphocytes in culture medium.
‡When acetaminophen (2 g/kg) was administered to rats having corn oil diet.

HIV: human immunodeficiency virus; NIDDM: non-insulin-dependent diabetes mellitus
anemia, metabolic acidosis and progressive neurological symptoms. On the other hand, only haemolytic anemia without 5-oxoprolinuria can be observed in patients with glutathione synthetase deficiency restricted to erythrocytes. The molecular basis of the glutathione synthetase deficiency, which is inherited in an autosomal recessive mode, has recently been clarified by Dahl et al. (1977). They have sequenced the glutathione synthetase alleles associated with enzyme deficiency and detected the mutations by direct sequencing of cDNAs and genomic DNA. Glucose-6-phosphate dehydrogenase is an enzyme necessary to renew the NADPH in cells, therefore indirectly related to GSH metabolism. If the level of the glucose-6-phosphate dehydrogenase activity is low in erythrocytes, NADPH levels will also be decreased. Then reduced GSH could not be regenerated in adequate amounts. Thus, reduced levels of GSH allow peroxides to accumulate in erythrocytes, and hemolysis can occur due to their oxidative effect on the lipids of the red cell membrane. Patients with glucose-6-phosphate dehydrogenase deficiency are sensitive to some drugs including antimalarial drug primaquine and also ingestion of the fava bean. Other enzyme deficiencies of GSH such as γ-glutamylcysteine synthetase, γ-glutamyl transpeptidase, and 5-oxoprolinase deficiencies can also be seen with various symptoms including haemolytic anemia, glutathionuria and 5-oxoprolinuria.

In addition to congenital enzyme deficiencies of GSH metabolism, disturbances in tissue thiol homeostasis have also been linked with several other clinical disorders including AIDS, diabetes, adult respiratory distress syndrome, cancer and neurodegenerative diseases such as Parkinson’s disease. Of them the ones associated with decreased tissue glutathione levels are shown in Table 1. Characteristically HIV infected individuals have decreased levels of acid soluble thiols, cysteine, and GSH in particular, in their plasma, epithelial lining fluid and leucocytes. Decreased glutathione levels in plasma and epithelial lining fluid of the lung in patients with AIDS may reflect a general glutathione deficiency in the extracelluar area. Elevated plasma glutamate lowers cellular GSH by inhibiting cystine uptake by cells. Flow cytometric studies have revealed that lipote treatment can increase cellular GSH levels on a dose-dependent manner by bypassing glutamate inhibition of GSH synthesis by reducing extracellular cystine to cysteine. Depleted lung glutathione level in patients with AIDS may have particular importance because of the opportunistic infections. In addition to AIDS, decreased epithelial lining fluid glutathione level is also found in patients with adult respiratory distress syndrome and idiopathic pulmonary fibrosis. Increased amount of H$_2$O$_2$ has been demonstrated in the expired air of patients with adult respiratory distress syndrome. Therefore, glutathione in the epithelial lining fluid may be consumed to scavenge this increased H$_2$O$_2$. On the other hand potentially significant connections between GSH and carcinogenesis have also attracted attention. The reactive ultimate carcinogenic forms of chemical carcinogens are electrophiles, and thus good candidates for detoxication by reactions catalyzed by GSH S-transferases. Decreased erythrocyte GSH in protein-energy malnutrition may be due to deficiency of amino acids, which are necessary for glutathione synthesis. Depleted plasma total glutathione level in cirrhosis has been attributed to impaired capacity of the GSH release from the cirrhotic liver. Helicobacter pylori elicits gastric mucosal cell damage by attracting phagocytes, which are producing reactive oxygen species. This may lead to decreased mucosal GSH levels in patients with Helicobacter pylori associated gastritis.

Autoxidation of glucose increases intracellular NADH/NAD$^+$ ratio and decreased GSH level are among the factors contributing increased oxidative stress in diabetes. Ciuchi et al. (1996) has reported decreased erythrocyte glutathione level which has been negatively correlated with plasma glucose and erythrocyte sorbitol levels. Therefore, they have proposed that enhanced rate of glucose metabolism through the polyol pathway may decrease glutathione level resulting in oxidative stress. In addition to decreased glutathione, lower activity of gamma-glutamylcysteine synthetase and thiol transport are also found in erythrocytes of diabetic patients. Treatment with an oral antidiabetic agent or insulin for 6 months has been restored those alterations. Even though decreased erythrocyte glutathione level is not affected, elevated malondialdehyde level has been decreased by constant intravenous glucose and continuous insulin perfusion maintaining normalglycemia for 3 days in erythrocytes of patients with
NIDDM. On the other hand, plasma thiobarbituric acid reactive substances and VO2max have been negatively correlated in insulin-dependent-diabetic patients at rest. In line with this clinical finding, endurance training has favourably decreased lipid peroxidation in kidney and vastus lateralis muscle and unregulated glutathione peroxidase activity in red gastrocnemius muscle in streptozotocin-induced diabetic rats in recent experiments. These experimental and clinical findings suggest that aerobic exercise and physical fitness may have a protective effect against oxidative stress in diabetes. Taken together, it seems that any treatment which maintains blood glucose level within the normal range is useful to decrease oxidative stress, which is linked with micro- and macrovascular complications in diabetes.

Decreased brain GSH levels have also been reported in neurodegenerative diseases such as Parkinson’s and Alzheimer’s diseases in which oxidative processes contribute to the pathology. GSH depletion in the brain created by intraventricularly administered BSO and dopamine in rats has been led to increased latencies to find the hidden platform in Morris water maze testing spatial learning and memory. These findings suggest that GSH may also have importance for the cognitive functions of the central nervous system. On the other hand, carbon monoxide exposure known to cause oxidative stress in brain has been increased plasma oxidized and reduced glutathione levels by releasing them from erythrocytes in rat.

Two questions arise when decreased GSH level is found in a disease: first, is the observed decrease of GSH cause or symptom of the disease? In the case of hereditary deficiencies of GSH synthesising enzymes, GSH deficiency is clearly the direct cause of the disease. The acidosis induced by 5-oxoproline accumulation as a consequence of GSH synthetase deficiency represents an indirect pathogenic factor. Second, is the extent of GSH decrease large enough in order to account for the symptoms of the disease? A 50% depletion of GSH still leaves more than 1 mM cellular GSH, and it is sufficient for GSH dependent processes to function. From a prophylactic or therapeutic standpoint of view, a compensation of the GSH loss is suggested before the critical lower threshold point (∼30% of normal) is reached. N-Acetylcysteine is suggested in the treatment of acetaminophen poisoning, which decreases tissue GSH levels.

Conclusion
It seems that glutathione metabolism still provides an extensive area of research to scientists in cell biology and exercise physiology thanks to its central role in antioxidant defense mechanism and in vitro and in vivo modulation by specific inhibitors for experimental purposes. Even though they are not common, it may be useful if paediatricians also take into account the deficiencies of the GSH metabolism in differential diagnosis of children with haemolytic anemias and progressive neurologic symptoms. In light of the recent evidence, it seems that increased oxidative stress accompanies various clinical disorders including AIDS, diabetes mellitus, adult respiratory distress syndrome, malnutrition, cirrhosis and Parkinson’s disease. Maintenance of the normoglycemia in diabetic patients is important to prevent oxidative stress. In addition, physicians treating these diseases may also consider to incorporate antioxidant therapeutics in their recipes to support antioxidant defense mechanism of the patient. Antioxidants may also be suggested for the prophylactic purposes in the elderly.

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