Assessment of Copper Nutritional Status

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Despite increased interest in the role of copper deficiency in clinical problems and an increased understanding of the physiological roles of copper, the diagnosis of a marginal deficiency has not been perfected. The use of nonstandardized procedures and the effects of factors other than copper nutriture have impeded identification of the "ideal" indicator of copper nutritional status in adult humans. The specific activity of copper enzymes, or of copper-containing enzymes in blood cells, such as erythrocyte superoxide dismutase and platelet or leukocyte cytochrome c oxidase, may be a better indicator of metabolically active copper stores than the serum concentration of copper or ceruloplasmin, because the enzyme activities are sensitive to changes in copper stores and are not as sensitive to factors not related to copper nutriture. A single index, such as serum copper concentration, is inadequate for assessing the total body copper nutriture of an individual and must be supported by corroborating evidence.

Indexing Terms: dietary copper/ceruloplasmin/superoxide dismutase/cytochrome c oxidase

In recent years there has been increasing interest in the role of copper deficiency in certain clinical conditions, particularly those experienced by young infants. However, many clinical issues remain unresolved despite an increasing understanding of the physiological role of copper. In 1979, Solomons (1) indicated that although there had been major advances in our ability to measure trace metal concentrations in body fluids and tissues and in metalloprotein enzymology, our fundamental clinical understanding of how to measure the trace mineral nutriture of an individual has lagged behind the analytical technology. Almost 15 years later, this observation still seems to hold true for most trace minerals. Here I discuss some of the difficulties and review several promising approaches in the clinical assessment of copper nutriture.

Clinical Relevance

Nutritional copper deficiency occurs in humans under a variety of conditions. Copper deficiency has been described in premature infants, neonates, and previously malnourished children (2,3); signs of deficiency include anemia, neutropenia, and skeletal demineralization. Copper deficiency in patients on long-term total parenteral nutrition (TPN) is also well documented (4-6). Clinical manifestations such as leukopenia, neutropenia, and hypochromic anemia unresponsive to iron supplementation appear only after several months in adult patients on TPN to which copper has not been added (4); serum copper and ceruloplasmin concentrations decline progressively in patients on un-supplemented TPN (7,8).

There is substantial evidence (9) that subclinical copper deficiency affects all stages of atherosclerosis and contributes to an increased risk of coronary heart disease. This concept is supported by both epidemiologic (10) and experimental studies. Abnormal electrocardiograms, hyperlipidemia, and blood pressure changes have been observed in both humans (11,12) and animals (13) experimentally depleted of copper; cardiac lesions and hypertrophy are consistent consequences of severe copper deficiency in animals (14). Low copper status also has been implicated as a risk factor in postmenopausal bone loss (15).

Much of the pathology of copper deficiency may be traced to metabolic defects involving various copper-containing enzymes. Table 1 summarizes some of the major cuproenzymes, their catalytic function, and the known or possible pathology associated with deficiency of each enzyme. Although specific pathology has not been established for all cuproenzymes, their activities are generally depressed by copper deprivation. For recent reviews on the biochemistry of copper, see refs. 16 and 17.

Unrecognized copper deficiency, or marginal deficiency, may be much more common than previously realized. Adult human copper dietary recommendations have been variously estimated between 1.2 and 2.0 mg of copper per day (18). Many diets, however, fail to provide this amount. Calculations based on surveys of ~850 individual diets from North America and Europe, in which copper content was measured by chemical analyses, indicated that 35% of diets provided <1.0 mg of copper per day (19), the approximate amount of dietary copper found to be insufficient in some short-term...

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1Nonstandard abbreviations: TPN, total parenteral nutrition; AAS, atomic absorption spectrometry; ICP, inductively coupled plasma emission spectroscopy; ENZ/RID Cp, specific enzymatic activity of ceruloplasmin (enzyme activity/immuno-reactive protein); and Cu-Zn-SOD, copper-zinc superoxide dismutase.
depletion experiments with humans. Surveyed hospital diets provided intakes of only 0.62–0.86 mg of copper per day (19).

Despite an increased understanding of the physiological roles of copper and an increased interest in copper deficiency in certain clinical conditions, our knowledge regarding the diagnosis of marginal copper deficiency is limited (20), particularly in adults. It has been difficult to demonstrate that copper deficiency occurs in adult populations by using conventional assays of copper status, such as plasma copper or ceruloplasmin concentrations; these do not always accurately reflect body copper stores (21). This was evident in several studies of experimental copper deprivation of men and women fed diets believed to be low in copper. Physiological changes, such as abnormal electrocardiograms (11), abnormal glucose tolerance (22), and blood pressure changes (12) that responded to copper supplementation, were noted, but conventional biochemical signs of copper depletion were largely absent or inconsistent (11, 12, 22–24).

Methods for Assessing Copper Nutritional Status

Plasma or Serum Concentrations of Copper

The concentration of copper in serum or plasma, the traditional marker of copper nutritional status, may be a relatively poor marker of short-term marginal copper status, but could be indicative of severely depleted copper stores (23). Low plasma copper concentrations are an early and consistent sign of copper deficiency in animals. Low plasma copper concentrations have been documented in patients after TPN with copper-deficient hyperalimentation solutions (4–8), in neonates, premature infants, and previously malnourished children with a dietary copper deficiency (2, 3), and in one man experimentally depleted of copper (11). Low plasma copper concentrations are also a manifestation of Menkes kinky hair syndrome (25), an inborn defect of copper metabolism. In all of the above cases, plasma copper concentrations were restored to normal by copper supplementation of the diet.

Under normal conditions, the concentration of copper in plasma is regulated by strong homeostatic mechanisms. Healthy, free-living individuals who were presumed to be consuming adequate amounts of copper maintained their plasma copper concentrations within a relatively narrow range of ±6.7–8.6% over periods of 2 weeks to 13 months (26, 27). Plasma copper concentration is significantly more variable (±20%) during experimental copper deprivation (23), and may not decrease significantly below reference ranges unless stores are depleted (11, 23).

Circulating copper concentrations are also sensitive to several factors not directly related to copper nutritional status. It is well established that women generally have significantly higher plasma or serum copper concentrations than men (21, 28), and that estrogen in the form of oral contraceptives increases plasma copper concentrations in younger women (1, 21, 28). Fischer et al. (28) and Nielsen et al. (29) reported significantly higher serum copper concentrations in postmenopausal women on estrogen replacement therapy. Other conditions that increase plasma copper concentrations include pregnancy, infections, and inflammation (1); rheumatoid arthritis is characterized by low serum zinc and high serum copper concentrations (30). Increased serum copper has also been reported immediately following myocardial infarction (31, 32) and in patients with dilated cardiomyopathy (33). Corticosteroids and corticotropin, in contrast, tend to lower plasma copper concentrations (1). Thus, conditions that increase plasma copper may be expected to obscure changes in copper status even during copper deprivation. Conditions that lower plasma copper concentrations must also be ruled out before a proper assessment may be made.

Atomic absorption spectrometry (AAS) is the method of choice for copper analysis. Copper concentrations in plasma or tissues are traditionally determined by flame AAS or by graphite furnace AAS (34). The advantage of flame AAS is speed of analysis, whereas the advantage of graphite furnace AAS is its sensitivity and smaller sample size. Methods recently have been developed for the rapid determination of copper in serum and urine by graphite furnace AAS with Zeeman effect background correction (35).

The last two decades have seen the introduction of the use of inductively coupled plasma emission spectroscopy

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Functional role</th>
<th>Known (or possible) consequence of deficiency</th>
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<tbody>
<tr>
<td>Ceruloplasmin</td>
<td></td>
<td>(Anemia, impaired Fe metabolism, impaired Cu supply to tissues)</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>Electron transport</td>
<td>(Cardiomyopathy, muscle weakness, growth failure, brain degeneration/neurological defects)</td>
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<tr>
<td>Superoxide dismutase</td>
<td>Superoxide radical decomposition</td>
<td>(Membrane damage, cell death)</td>
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<tr>
<td>Lysyl oxidase</td>
<td>Cross-linking of collagen and elastin</td>
<td>Vascular rupture, osteoporosis, loose skin and joints</td>
</tr>
<tr>
<td>Tryrosinase</td>
<td>Melanin production</td>
<td>Lack of pigmentation</td>
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<tr>
<td>Dopamine-β-hydroxylase</td>
<td>Catecholamine metabolism (dopamine-- norepinephrine)</td>
<td>(Neurological defects, cardiac hypertrophy)</td>
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<tr>
<td>Amine oxidase</td>
<td>Oxidizes mono-, di-, and polyamines</td>
<td>—</td>
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<tr>
<td>Factors V and VIII</td>
<td>Blood coagulation</td>
<td>(Bleeding and clotting disorders)</td>
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<tr>
<td>Thiol oxidase</td>
<td>Disulfide-bond formation</td>
<td>(Steely wool, Pili torti)</td>
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(ICP) for the analysis of mineral and trace elements in biological samples (36). The advantages of ICP analytical techniques include: (a) simultaneous multielement analysis; (b) absence of solute-vaporization-type interferences; (c) the dynamic range of linear calibration generally covering four to five orders of magnitude of concentration; and (d) the detection limits and sensitivities typically better than flame AAS. Techniques based on the use of ICP for the routine measurement of copper and other mineral and trace elements in urine and serum are described by Nixon et al. (37).

Several colorimetric methods for measuring serum copper, with use of a wide variety of ligands, have been described over the past 70 years. For more recent techniques that may be applied to autoanalyzers, see refs. 38 and 39. However, most colorimetric procedures are generally more prone to interferences and tend to lack the sensitivity and specificity found with AAS or ICP techniques.

Ceruloplasmin

Most of the changes in plasma copper concentrations noted above are associated with changes in the cuproenzyme ceruloplasmin (EC 1.16.3.1). It is often stated that this protein accounts for >80% of the copper in plasma. However, more recent studies suggest that ~60–72% of plasma copper is bound to ceruloplasmin (40). Both the enzyme activity and the amount of immunoreactive protein are higher in women than in men, and higher in pregnant women and in individuals taking oral contraceptives (estrogen) (21, 28, 29). Since ceruloplasmin is an acute-phase protein, it is also increased in cases of acute or chronic infection (41) or inflammatory stress (42). Ceruloplasmin is lowered in malnutrition, nephrosis, Menkes syndrome (43), Wilson disease (44), and chronic hepatitis (45). The enzymatic activity of ceruloplasmin has been found to be depressed in some men and women fed diets low in copper (23, 46); the activity returned to normal when copper was supplied to the diet. However, immunoreactive ceruloplasmin was not affected in these studies (23, 46). It is likely that a copper-free apoceruloplasmin or an inactive form of ceruloplasmin with a reduced number of copper atoms was present during the copper deprivation (46, 47). Recent studies have indicated that the specific enzymatic activity (the ratio of enzyme activity to immunoreactive protein, ENZ/RID Cp) may be a better indicator of copper status than either the enzyme activity or immunoreactive protein alone (21). ENZ/RID Cp is sensitive to copper status (46) and is inversely related to autonomic blood pressure response during copper deprivation in young women (12). It is not influenced by nondietary factors such as gender or hormone use (21).

Serum ceruloplasmin concentration has been determined by measuring its oxidase activity (48, 49) and by nephelometry (50), radial immunodiffusion (51), or immuno-electrophoresis techniques (44). Measurement of the oxidase activity with p-phenylenediamine may be subject to interferences by certain anions and the oxidation product of p-phenylenediamine (52). Discrepancies have been noted between radial immunodiffusion and various nephelometric assays (50). These discrepancies could be related to different sources of antibodies used in the assays (50). It has also been indicated that chromatography of crystalline ceruloplasmin produces several immunoreactive components (53), and changes in antigenicity of ceruloplasmin has been observed with loss of copper from the protein (54). However, either or both enzymatic or immunochemical methods can provide clinically useful data if reference ranges are established in each laboratory and if conditions used for analysis are carefully maintained. This also applies to the other copper-containing enzymes superoxide dismutase and cytochrome c oxidase.

Superoxide Dismutase

Copper–zinc superoxide dismutase (EC 1.15.1.1; Cu-Zn-SOD) is located in the cytosol of most tissues and is an integral part of the body's defense mechanism against the consequences of oxygen metabolism. Dietary copper deficiency in several different species of animals leads to low Cu-Zn-SOD activity in most tissues, including lungs, aorta, liver, and erythrocytes; the activity in erythrocytes tends to parallel the copper concentrations and activities of various copper-containing enzymes in tissues (55). These observations in animals have prompted several investigators to use Cu-Zn-SOD activity as an index of copper status in studies with humans. Okahata et al. (56) observed lowered Cu-Zn-SOD activity in a copper-deficient, 7-month-old child, and Uauy et al. (57) reported low Cu-Zn-SOD activity in 17 infants recovering from malnutrition and receiving marginal copper intakes. In all cases, activity returned to reference levels upon supplementation with copper. Some studies of experimental copper deprivation of adult humans have demonstrated that Cu-Zn-SOD activity is decreased during copper deprivation and may be restored when copper is added to the diet (11, 23).

In contrast to serum concentrations of copper or ceruloplasmin, erythrocyte Cu-Zn-SOD activity does not seem to be affected by age, gender, or hormone use (21, 28, 58). Recent studies, however, have suggested that some conditions that produce an oxidative stress tend to increase Cu-Zn-SOD activity, even during periods of low copper intake (59, 60). Lukasaki et al. (61) showed that erythrocyte Cu-Zn-SOD activity is elevated in competitive swimmers during training. It was hypothesized that this elevation was a functional adaptation to increased oxygen utilization during increased aerobic physical training.

Most available assays for Cu-Zn-SOD activity are based on the indirect measurement of activity (34). This type of assay consists of a superoxide-generating system and a superoxide indicator that is measured spectrophotometrically. Addition of Cu-Zn-SOD inhibits the absorption change of the indicator in a degree proportional to the amount of the enzyme present. Flohé and Ottling (62) and Beyer and Fridovich (63) list several indirect assays of Cu-Zn-SOD activity; no single method seems to be favored, but methods based on the reduction of
cytochrome c or on the reduction of nitroblue tetrazolium are widely used. Many of these assays are prone to interferences (64) and caution is indicated in selecting the appropriate procedure. A method based on the autoxidation of pyrogallol (65) seems to be relatively free of interferences. Many of these procedures have lent themselves to automation (66). Recently, methods based on the detection of superoxide by chemiluminescence (67), the direct measurement of changes of superoxide (68), or the formation of hydrogen peroxide (the product of conversion of superoxide by superoxide dismutase) (69) have been reported. Immunochemical measurements of Cu-Zn-SOD have been described (70). However, they are mainly still in the developmental stage and have not been widely applied to clinical situations. As with ceruloplasmin, in spite of the plethora of methods that provide apparently different numbers and different definitions of "units," analysis of Cu-Zn-SOD can provide clinically useful data if reference ranges are established in each laboratory and conditions used for analysis are carefully maintained.

Cytochrome c Oxidase

Low tissue activity of cytochrome c oxidase (EC 1.9.3.1), the terminal oxidase enzyme in the electron transport chain, is an early and consistent sign of copper deficiency in animals (24). Defects in cytochrome c oxidase activity can cause neurological, cardiac, and muscle disease when the activity is only ~50% of normal (71). Recent studies have indicated cytochrome c oxidase deficiencies in children with manifestations of central nervous system or neuromuscular disease combined with hyperlactemia (72), and in individuals with Leigh syndrome, a neurological disorder (73). It has also been reported that Menkes disease, which causes a severe copper deficiency in children and infants, markedly lowers leukocyte cytochrome c oxidase activity (74). Platelet and leukocyte cytochrome c oxidase activity in young women was reduced by low copper intake, whereas erythrocyte Cu-Zn-SOD activity was unaffected (46); platelet cytochrome c oxidase was the most sensitive indicator of copper deprivation in a recent study with postmenopausal women (75). Studies with rats (76, 77) have shown that platelet and leukocyte cytochrome c oxidase activity is sensitive to copper status; the cytochrome c oxidase activity in platelets correlates with copper concentrations in liver, an established marker of copper status in animals. These observations indicate that platelet cytochrome c oxidase activity is a useful indicator of copper status in humans and merits further investigation. Cytochrome c oxidase activity in platelets and mononuclear leukocytes is higher in older adults than in young adults, but is not affected by gender or hormone use (21). However, there seem to be large subject-to-subject variations, the enzyme is fairly labile, and cytochrome c oxidase assays are sensitive to minor variations in technique—all of which may limit the usefulness of this enzyme, at this time, as a general indicator of copper status in adults.

Most methods for determining cytochrome c oxidase activity in tissues and blood cells are based on the spectrophotometric analysis of the oxidation of ferricytochrome c (34, 78, 79). A microassay has been described that utilizes a coupled reaction between cytochrome c and 3,3'-diaminobenzidine tetrachloride in microwell plates (80). This method has the advantage over the traditional spectrophotometric method in that only a small number of cells are used and the procedure is much faster.

Summary Comments

The diagnosis of marginal copper deficiency, particularly in adults, has not been perfected, largely because of the use of nonstandardized procedures and because of the effects of factors other than copper nutriture. It is likely that the activity of copper-containing enzymes in blood cells, such as cytochrome c oxidase in platelets and superoxide dismutase in erythrocytes, are more reflective of metabolically active copper and copper stores than the plasma concentration of copper or ceruloplasmin (21). A change in, or a lower concentration of, a single index may be inadequate for assessing total-body nutriture of an individual and must be supported by corroborating evidence.

References