Review article

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BLUE MOONLIGHTING IN THE IMMUNE RESPONSE: ROLES OF COPPER AND CERULOPLASMIN IN THE PATHOGENESIS OF INFLAMMATION AND IMMUNE-MEDIATED DISEASES

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Increase in serum copper levels and/or its main blood carrier - ceruloplasmin (Cp) is a constant finding in some human diseases. One of the best-known roles of Cp is the regulation of cellular iron efflux in situations of hypoxia. Nevertheless, copper and Cp are involved in multiple physiological processes, such as redox balance, regulation of transcription factors, neuronal growth, some immune functions: microbicidal activity, cytoprotective barrier, lymphocyte proliferation, etc. Ceruloplasmin is an acute phase reactant and therefore its levels increase in conditions of acute infections or inflammation. Also, copper dyshomeostasis has been clearly established in many inflammatory autoimmune diseases, malignancies, neurological and obstetric disorders. Changes of copper and Cp metabolism are reported in the pathogenesis of diabetes mellitus and cardiovascular diseases. Besides, alterations of serum copper can be utilized as a prognostic and predictive biomarkers. However, interpretation of these data is not fully recognized in the routine clinical practice. Therefore, the aim of our work is to review current knowledge and recent evidence about the roles of copper and Cp as a part of the immune response in the etiopathogenesis of multiple diseases and present the usefullness of interpretation of their altered levels.

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Introduction

Increased levels of copper (Cu) in the blood or hypercupremia is a constant finding in certain human diseases, as well as some physiological states, which is often underestimated and left out from interpretation. However, assessment of serum copper levels (SCL), or its main blood carrier - ceruloplasmin (Cp), may help in guiding a diagnostic protocol.

Ceruloplasmin and copper are involved in multiple physiological processes, cellular mechanisms, and signaling pathways. Ceruloplasmin has been called a promiscuous or moonlighting enzyme, mainly because there is a multitude of substrates it can act on. Moreover, copper may bind to the sites on Cp other than the active site, creating additional accessory functions. One of the best-known Cp functions is ferroxidase activity by which it helps iron efflux from the cells in situations of hypoxia. It also performs oxidation of biological amines, lipids, and oxidative inactivation of NO. Together with zinc and selenium, copper displays an important role in the immune system. Human macrophages use copper as a bactericidal and fungicidal agent in phagosomes, which is achieved by its marked accumulation during cell activation. Fluctuations in the copper concentration influence the activity of a transcription factor NF-κB, as well as hypoxia-inducible factor 1 alpha (HIF-1α). Copper is a catalytic cofactor of the two important anti-oxidative enzymes, cytosolic and extracellular Cu(II)/Zn(II)-superoxide dismutases (SOD1 and SOD3). Besides, copper is needed for axonal outgrowth, synapse integrity, neuronal activation, and motor neuron maintenance (1-8).

Nevertheless, copper may be potentially toxic and cause dysregulation of physiological processes. Its involvement in oxidation-reduction reactions requires close regulation because through the shift of its redox states it can generate damaging hydroxyl radical and interfere with the function of other intracellular metals. It may displace other metals from its cognate organic ligands, leading to inappropriate

changes in protein functions. This process is explained by the Irving-Williams series of the relative stabilities of complexes formed by divalent first-row transition metal ions, which increase across the period to maximum stability at copper (1, 3, 7).

Physiologically, females have slightly higher copper levels than men, which additionally increase during pregnancy. Reactive or secondary changes in the concentration or availability of copper mostly occur in immunological and inflammatory conditions. Also, total copper serum concentrations frequently increase in cancer patients. Several studies estimated its values in the diagnostics and staging, as well as a prognostic marker in various diseases (7, 9, 10).

The objective of this scoping review was to summarize current knowledge and recent evidence about the roles of copper and Cp as a part of the immune response in the etiopathogenesis of multiple diseases that have an immune or inflammatory background. We also tried to present the usefulness in the interpretation of their altered serum levels in diagnostics.

Basic physiological concepts of copper metabolism

Copper is an essential trace element (oligoelement), with many natural sources and diverse metabolic functions. It participates in many biochemical reactions and structures of many biomolecules. Copper ions can exist in a reduced (Cu+) or an oxidized (Cu2+) state. In the reduced form, it is mainly found in intracellular space, as oxidized in fluids, and in redox enzymes it shuttles between the two forms. This gives copper an affinity for binding to the different side-chains of amino acids and thus provides the ability to interact with many proteins. Therefore, it is a part of proteins such as transcription factors, transporters, chaperones and storage proteins, oxidases, monooxygenases and oxidoreductases (ascorbate oxidase, dopamine-monooxygenase, lysyl oxidase, phenylalanine hydrolase, tyrosinase, *e.g.*), electron transfer proteins (cytochrome c oxidase), free radical scavengers (Cu, Zn superoxide dismutase - SOD), and others (1-3, 7).

Copper is transported across the apical membrane of enterocytes by a copper transport protein 1 (Ctr1), a redundant high-affinity copper cellular transporter, and can transfer via a divalent metal ion transporter 1 (DMT1). Then it becomes transferred by the Atox1 Cu chaperone to the ATP7A enzyme (a Cu-transporting P-type ATPase) at the basolateral membrane into the portal circulation and liver. It is stored in the liver, mainly in the mitochondria, specifically in the complex IV of the respiratory chain. When in excess, it can be sequestrated by metallothioneins or glutathione. ATP7A also participates in copper transport across different polarized cell layers, as well as intracellular organelles such as Golgi apparatus and phagosomes. Another ATPase ATP7B is a transmembrane copper transporter specific for the liver, which aids in copper loading on apo-ceruloplasmin and export of its excess into the bile (1-4, 7). From a clinical point of view it is important to say that it takes a few weeks for Cp to reflect changes of copper in diet (11).

Ceruloplasmin is a multifunctional protein that tightly binds 6 atoms of copper per molecule. It is recognized as a carrier of most copper in the blood (about 40-70%), which it transports from the liver to the distal tissues. A smaller percent of copper is delivered by albumin, α2-macroglobulin (transcuprein), and several other small molecules. Ceruloplasmin belongs to the α2-glycoprotein fraction and has two molecular Cp isoforms: secreted and a membrane glycosylphosphatidylinositol (GPI)-anchored form. Secreted Cp is a plasma oxidase, primarily known for its potent Cu-based ferroxidase activity, which makes it essential for iron homeostasis. In the presence of O2 Cp may convert iron from ferrous (Fe2+) to ferric ion (Fe3+), therefore assisting and enhancing iron efflux from cells' storage through the ferroportin and iron-binding to plasma apotransferrin or apoferritin. The iron release from macrophages is specifically induced under conditions of hypoxia, to be used for erythropoiesis (10-12). The Cp gene contains a response element for the alpha subunit of hypoxia-inducible factor (HIF1-alpha) transcription factor, the main regulator of cellular response to hypoxia. In this context, Cp is thought to be reserved for iron regulation due to relative hypoxia. Besides hypoxia and apotransferrin, the process requires an available intracellular "labile iron pool" and a low extracellular free iron level, which creates a negative iron gradient. Ferroportin is the only known export pathway of intracellular non-heme-associated iron (10, 13).

Monocytes and macrophages additionally display the GPI-membrane-linked Cp in lipid rafts, with increased expression and colocalization with ferroportin after iron treatment. This GPI-anchored form is encoded by an alternatively spliced variant of the Cp gene (12-15). Another multicopper protein homologous to Cp is hephaestin. However, it is a transmembrane ferroxidase mostly found in enterocytes of the villi and is responsible for transporting dietary iron (16). Besides, a few more molecules are determined to bind and use copper, such as zyclopen, native prion protein (PrPC), clotting factors V and VIII, etc (4, 10, 17).

However, all physiological roles of Cp are still not well defined. Ceruloplasmin is also an acute-phase reactant, meaning that its levels increase during inflammation, infection, malignancies or trauma. Due to the expression of ATP7B copper transporter, lymphocytes and macrophages are not likely to accumulate copper (18). However, ATP7A protein levels rise with macrophage (M1) activation and it translocates to the phagosomes, where increases copper content aiming for the preparation for antimicrobicidal functions. Concurrently, there is an increase in Cp expression (1).

Ceruloplasmin is involved in the regulation of redox reactions, but its roles are still not defined well, because depending on a situation it was determined both anti-oxidant and pro-oxidant.

Ceruloplasmin is considered the main extracellular radical scavenger that inhibits a variety of oxidative reactions, such as superoxide dismutation, peroxidation, prevents spontaneous oxidation of

Fe2+ (the Fenton reaction), as well as consequent damage to the biomolecules. It also performs oxidative inactivation of NO and some biogenic amines. Ceruloplasmin oxidates NO to NO+ with subsequent hydration to nitrite, its bioactive reservoir (5, 10, 19, 20).

Average SCL for adults is estimated as from 11 up to 22 μmol/L (70-140 μg/dL), with slightly higher levels in females - 12.5-24 μmol/L (80-155 μg/dL). Infants from birth to the 6th month have copper levels between 3-11 μmol/L, up to 6 years of 14-30 μmol/L, after which they decline to 13-25 μmol/L. Normal values of copper in 24h urine are < 60 μg/24h (1.0 μmol/24h) in adults, while significantly higher levels are present in Wilson disease, that is > 200 μg/24h urine (> 3.0 μmol/24h). Average Cp serum levels are 20-60 mg/dL (0.93 to 2.65 μmol/L). Besides, many medications are known to increase Cp levels, e.g. anticonvulsants (2, 10, 21-23).

Primary disorders of copper and Cp metabolism

Inherited disorders of copper homeostasis are rare and commonly affect Cp or other proteins involved in the copper transfer and/or excretion system. They are characterized by copper accumulation in specific organs which, however, creates its deficiency in the remaining parts of the body. Mutations in the gene of Cp may cause aceruloplasminemia with resulting iron accumulation and related tissue damage. Menkes diseases and Wilson disease (WD), or hepato-lenticular degeneration, are genetic disorders with mutations in the genes for ATP7A and ATP7B, respectively. The mildest form of Menkes disease is occipital horn syndrome. Disruption in the ATP7A gene leads to the copper sequestration into the intestine and kidneys, while ATP7B defect causes deficiency in biliary copper excretion and its accumulation, dominantly in the liver and brain (basal ganglia), with their progressive damage (1, 8, 24-26). Signs and symptoms are related to the deficiency in enzymes that require copper for its function, such as lysyl oxidase and dopamine-β-hydroxylase. These include connective tissue abnormalities, neurodegeneration, dysautonomia, ataxia, dystonia, psychiatric disorders, liver damage, amenorrhea and subfertility, anemia, etc. (8, 24-28).

These conditions are usually associated with lower than normal Cp blood levels (< 20 mg/L). Therefore, diagnostic criteria for WD are low serum Cp and copper levels together with a high urine copper excretion. It is important to start copper chelation therapy, along with zinc substitution, early in the onset of WD, because the therapy is ineffective in advanced stages. There are ongoing studies over genetic therapy for Menkes disease, because chelation therapy is ineffective in this case and the disease is lethal (1, 26, 27).

In a mouse model of WD, several important observations were made regarding copper handling in hepatocytes. Copper availability within a cell must be carefully controlled to avoid damaging copper-dependent redox cycling. One of the first responses to the copper overload was a downregulation of Ctr1, Cu-influx protein, and potential engagement of copper-export mechanisms. This was followed by a dynamic change in cellular metabolism with the formation of intracellular and extracellular copper deposits and enhanced accumulation in surrounding cells, especially lymphocytes and macrophages. It is important to note that although copper levels increased in the immune cells, this redirection seemed not to cause pathological copper accumulation or disturb cellular metabolism, likely due to the existence of other copper transporting enzymes. Besides, concomitant with the dynamics of copper compartmentalization, specific changes occurred in the metabolic pathways of hepatocytes. Importantly, during all evolution stages, significant retardation of lipid metabolism and oxidative-reductive processes was present (18).

Reactive changes of copper and Cp metabolism

Copper-containing proteins show a range of roles in support of metabolic and homeostatic processes. Therefore, it is not surprising that various pathological derangements can create unbalance in copper distribution. Although hepatocytes are the main producers of Cp, other cell types are capable to produce and secrete Cp as well, such as activated macrophages and mononuclear cells during inflammation, lymphocytes, astrocytes, leptomeningeal cells, choroid plexus of the brain, retina, kidneys, spleen, the epithelial lining of the uterus, placenta (syncytiotrophoblasts), mammary epithelial cells, Sertoli cells, adipocytes, etc. (5, 10, 14, 23, 29, 30). Besides copper itself, the main regulators of Cp expression are hypoxia, inflammatory cytokines, and female sex hormones (estrogens and progestagens). Hence, healthy females have slightly higher SCL and Cp compared to males (10). Regarding their cellular sources, increased copper and Cp plasma levels are commonly expected in inflammation, infections, lymphoproliferative and autoimmune disorders, pregnancy or contraceptive use, hepato-biliary disorders, malignant and some neurologic diseases (10, 30-33).

Copper and Cp in acute inflammation and infection

Increased SCL and Cp levels are common in infection and inflammation, because they are a part of the acute phase response. As an acute phase reactant, Cp serum concentration and activity increase nearly double or approximately up to 900 μg/mL. Accordingly, the plasma levels of copper increase, while the levels and availability of iron decrease, following the change in its regulatory proteins (ferritin, transferrin, hepcidin, etc.). The acute phase response is largely mediated by proinflammatory cytokines (IL-1, IL-6, TNF-α, and IFN-γ) (10, 15, 20, 23). And IL-6 seems to be the main cytokine for stimulation of Cp gene transcription, as there are three IL-6 response elements in the enhancer and promoter regions of its gene. Besides, Cp gene

expression can be induced by bacterial lipopolysaccharide (LPS) during inflammation (10, 34).

Peripheral blood lymphocytes constitutively express both isoforms of Cp (soluble and GPI-linked membrane form). Importantly, the greatest Cp expression was found on natural killer (NK) cells (CD3-CD16+/CD56+ lymphocytes) compared to other major lymphocyte subsets (10, 14, 23). In both infection and inflammation, active proliferation of lymphocytes is accompanied by the increase in serum Cp concentration. There is also speculation that the Cp increase might additionally originate from the direct secretion and/or surface shedding of Cp from peripheral blood leukocytes. Solubilization of GPI-linked proteins from the surface of activated NK cells has been described and was mediated by the actions of matrix metalloproteases (23, 35). Ceruloplasmin surface expression and shedding following lymphocyte activation are hypothesized to provide a part of the cytoprotective barrier by utilizing the Cp antioxidant capacity (10).

As already mentioned, upon activation, macrophages actively accumulate copper into the phagosomes, where it adds to the microbicidal defense (1). And vice versa, the copper deficiency was shown related to decreased respiratory burst and microbicidal activity of phagocytic cells (23). Free copper ions are prone to oxidation and reduction by which they may propagate the formation of reactive oxygen species (ROS) (20). In addition, when stimulated by LPS under inflammatory conditions, Cp potentiates inducible NO synthase (iNOS) activity and production of NO in microglial cells (10).

Moreover, changes in the copper concentration influence the activity of NF-κB and induce Golgi-complex-independent secretion of interleukins and cytokines, such as IL-1 (4).

Ceruloplasmin may cause both anti- and prooxidant effects on polymorphonuclears (PMN). Particularly, it reduced spontaneous and inflammation-induced accumulation of superoxide radicals, but also triggered a rapid increase of intracellular oxidation products (20). Ceruloplasmin acts as a potent endogenous inhibitor of neutrophil myeloperoxidase (MPO), some serine proteases, and forms complexes with other plasma proteins during inflammation, such as lactoferrin (22, 31, 36). Myeloperoxidase is a potent oxidant capable to exacerbate oxidative stress (OxS) in inflammation, while Cp was demonstrated to inhibit MPO production of hypochlorous acid by about 50% (20, 31). However, in the situations of severe inflammation, such as sepsis, Cp is overwhelmed and destructed by hydrogen peroxide and proteases. In addition, tyrosine residues of Cp can be nitrated in OxS by peroxynitrite (generated by MPO) which declines its ferroxidase activity. Its destruction is followed by the release of copper ions that further provoke the formation of $OH^{\cdot}$ and amplify the production of ROS. Nevertheless, it is suggested that leukocytes may actually use this reaction in order to enhance the production of $OH^{\cdot}$ in the acidic environment at the focus of inflammation as a part of the antimicrobial reactions (6, 31, 37). Interestingly, antineutrophil cytoplasmic autoantibody (ANCA) directed to MPO may impede Cp from binding to and inhibiting MPO, thereby promoting a prooxidant state (31). Conversely, lactoferrin is a multifunctional protein, actively secreted by PMN leukocytes. It is an important protector of Cp, capable of binding free Cu ions and thus limiting Cp fragmentation by hydrogen peroxide. This effect depends on the pH level and is being most efficient at 7.4. Overall, Cp interactions regulate local ROS generation during acute inflammation with concomitant protection of adjacent cells from collateral damage (10, 37).

An interesting finding is that Cp-GPI at the surface of human peripheral blood leukocytes (PBL) does not correlate with serum Cp levels, but correlates with ferritin levels (10, 14, 23). Recent studies show that ferroxidase activity is needed in cells expressing Cp-GPI to stabilize ferroportin molecules at the cell surface. Ferroportin becomes degraded in the absence of Cp or when there are sufficient levels of peptide hepcidin (5, 38). Although Cp stabilizes ferroportin and has an antagonistic effect on hepcidin, its raised levels in inflammation are not correlated with iron levels, but the opposite. During inflammation, iron needs to be sequestrated away from pathogens and as a substrate for oxidative stress. Thus, it seems that the action of Cp is reserved for situations of relative hypoxia and iron deficit. In addition, the same as to Cp, the expression of hepcidin is increased by inflammatory cytokines, especially IL-6. Hepcidin controls surface expression of ferroportin 1, by inducing its downregulation and intracellular degradation. Hypoxia, on the other hand, represses hepcidin synthesis (unlike Cp) through the HIF-2α factor (10, 16).

Another important role of Cp is in the tuning of neutrophil apoptosis, which is an essential step in the resolution of acute inflammation. Intact copper-containing Cp and partially proteolyzed forms inhibit delayed spontaneous neutrophil apoptosis, with the intact form showing a pro-survival activity. However, proteolytic forms display a potent TNF-α-induced activation of apoptosis (20).

Ceruloplasmin and copper effects and their regulatory enzymes are engaged during defense against infections caused by bacteria, viruses, and other microorganisms. For example, there are several points where copper metabolism interferes with Influenza A virus (IAV) survival and replication: holo-ceruloplasmin binds to IAV decreasing its infectivity and acting as a trap for virions at early stages, fluctuations in copper concentration reduce IAV reproduction, maintenance of SOD1 function hinders virus replication, etc (4). Apparently, effective replication of many viruses requires an enhanced cellular ROS production, and IAV activity targets the host cell antioxidant defense, among others by significant suppression of SOD1 gene transcription (4).

Interestingly, Cp is significantly increased in exudative pleural effusions compared to other acute phase proteins. Copper levels were significantly higher in both benign and malignant exudates compared to transudate due to fluid overload, and correlated with zinc and manganese levels (39, 40). A study found comparable sensitivity and specificity measures of pleural fluid Cp, and its ratio to the serum Cp, compared to the standardly used Light's

criteria, as well as positive predictive value (98%) of pleural fluid Cp (at ≥ 13.34 mg/dl) and the ratio (at ≥ 0.37) (40).

Copper and Cp in chronic inflammation

Alterations in copper and Cp metabolism are determined in a number of disorders with chronic inflammation and/or immune dysregulation, such as diabetes mellitus (DM), cardiovascular diseases, inflammatory bowel disease (IBD), systemic autoimmune connective tissue diseases, lymphoproliferative disorders, and other (4, 20, 30, 31, 41-44).

Diabetes mellitus and obesity

It is well known that chronic low-grade inflammation is implicated in the pathogenesis of DM type 1 and type 2, which is promoted by obesity and associated with insulin resistance and metabolic dysfunction. An activated immune response may be considered a common antecedent as well as a factor associated with the development of complications in type 2 DM (30, 45, 46). Significantly higher levels of Cp were determined in newly diagnosed type 1 and type 2 DM patients compared to controls (40.69 ± 9.9, 45.05 ± 9.0, and 26.95 ± 4.1 mg/dl, respectively). Interestingly, Cp was markedly reduced after 5-year treatment with oral hypoglycemic drugs (25.73 ± 9.94 mg/dl) (46). In another study, Cp level correlated positively with fasting glucose in healthy subjects along with CRP, however it did not predict the risk of DM incidence (45).

Obesity leads to increased adipocyte oxygen consumption that is due to the elevated free fatty acids level and availability. Increased adipocyte lipolysis results in uncoupled mitochondrial respiration and leads to a state of relative cellular hypoxia that triggers greater expression of HIF-1α and downstream pro-inflamamtory signaling with chronic tissue inflammatory response. Besides, HIF-1α is indicated as a key factor in adipose tissue macrophages (ATM) accumulation and functional change by stimulating chemokine and leukotriene-B4 production (30, 47).

Accumulated ATMs in obese adipose tissue are secreting most of the inflammatory cytokines and are dominant in governing metabolic dysfunction and insulin resistance. In healthy subjects, most of the resident ATM display anti-inflammatory M2-polarization with Th2 supporting effector functions. However, following an adipocyte-related rise in chemokine production ATMs undergo proliferation and phenotypic switching to the M1-like polarized pro-inflammatory state, along with increased recruitment of blood monocytes (M1), neutrophils, and Th1 cells (30, 48).

Differently polarized macrophages differently handle iron and express Cp gene. M1 cells exhibit potent anti-microbial properties with a relatively sealed intracellular iron content, but have a high expression of the Cp gene. On the other hand, M2 cells have ability to recycle iron, higher iron internalization, but downregulated Cp expression (49-51). Therefore, elevated Cp synthesis in M1 cells presumably serves for immune functions and anti-oxdidant defense rather than the iron metabolism. Given the HIF-1α response elements in the Cp gene and increased synthesis in M1 cells, elevated Cp and copper levels are following pro-inflammatory conditions preceding DM.

Higher serum Cp levels were determined in patients with arteriosclerosis. Dysregulation of iron handling and its retention in macrophages promotes the formation of foam cells in atherosclerosis. A study revealed decreased synthesis and activity of Cp in LPS-activated macrophages in the presence of iron and Ox-LDL, suggesting disturbed protective effects of Cp (52). Also, in prolonged inflammatory environment Cp may be submitted to degradation and release of Cu ions, which exhibit pro-oxidant features. Lipid metabolism seems very sensitive to copper imbalance. In this setting, enhanced oxidation of LDL particles had been related to the increased Cp levels, creating additional risk for the development of atherosclerosis (5, 18, 46). By utilizing cell-derived superoxide anion, Cp was shown to be a potent catalyst of LDL oxidation in several cell models (53). LDL contains a few classes of Cu(2+) binding sites, some of which are promoting lipid peroxidation during the propagation phase of atherosclerosis. Several proposed mechanisms explain the copper's ability to modify LDL, but the precise initiating reaction is unresolved (54).

Cardiovascular diseases

Ceruloplasmin has been associated with cardiovascular diseases especially myocardial infarction (MI). It was assessed as a risk factor predicting MI and related cardiovascular complications. Ceruloplasmin and SCL increase transiently after MI, which is consistent with the acute phase response to trauma (6, 11, 42, 53, 55).

Higher Cp levels were observed in patients with ST-elevation myocardial infarction (STEMI) within 12h after onset of chest pain compared to healthy controls (40.1 ± 9.7 vs. 31.4 ± 7.6 mg/dl). These levels significantly correlated with CRP values on the hospital admission, inversely correlated with left ventricular ejection fraction (EF), and were even the most significant marker of ensuing acute heart failure. Ceruloplasmin levels return to about normal after four weeks following infarction. Therefore, a rise in plasma Cp along with other inflammation-sensitive proteins seems to provide a short-term prognostic relevance in patients with systolic dysfunction after acute MI (42).

Most studies so far confirmed a direct relationship between higher serum Cp levels and the incidence of coronary heart disease (CHD) (11). There was a continuous increase in the risk of CHD with increasing Cp, in the middle-aged patients with dyslipidemia (34.9±86 vs. 31.7 ± 77 mg/dl, in controls, $p < 0.001$). The risk was doubled in the highest Cp tertile with an odds ratio of 11.3 in patients with low HDL cholesterol. Besides being determined as an independent risk factor for CHD, its levels were associated with high serum oxidants and decreased anti-oxidant status (11, 56).

Moreover, Cp concentration in CHD was found independently associated with increased risk of

death from cardiovascular and all causes (11, 57, 58). Elevations of CRP and/or Cp were found significantly related to subclinical myocardial necrosis (troponin I < 0.03 ng/mL) in nearly 4000 stable cardiology patients undergoing coronarography. Interestingly, the necrosis was not accompanied with the markers of leukocytes activation but was with the reduction in systemic anti-oxidant enzyme activity, suggesting that pathophysiologic process may be partially independent of leukocyte-mediated action on atherosclerotic plaques. This prodromal subclinical necrosis correlated with a higher long-term risk of major adverse cardiovascular events (MACE) (57). Patients with higher Cp (at 22.0 mg/dL) and MPO (at 322 pg/mL) showed the highest risk of future MACE (58).

Therefore, serum Cp value is a strong candidate for a group of so-called other modifiable non-traditional risk factors which could help to predict some CHD events (11). It seems that elevations of Cp and copper are due to both the inflammation and OxS, which underlie the development of CHD, and therefore may be used as surrogate markers of these processes. Nevertheless, in situations of the overwhelming OxS they could become pro-oxidants and add to the cell damage.

Additionally, copper is lost from the heart during myocardial ischemia. Correspondingly, SCL reached the highest level around the 10^{th} day and gradually recovered until the 21^{st} day. The changes were partially related to the upregulation of a copper transport chaperone - COMMD1 (60).

Investigations of chronic cardiac disorders observed somewhat different results. Particularly, Cp and copper were not found as pronounced markers in these disorders. In patients with chronic heart failure (HF) copper concentration was not significantly correlated with the left ventricular ejection or parameters of the diastolic left ventricular function (61, 62). However, there are some opposite findings, several reports found significant change in Cp levels in chronic cardiac patients. In the study of Cabassi et al. (63) patients with chronic stable HF presented significantly higher levels of Cp (2419 ± 523 vs. 2118 ± 478 nmol/L), copper (21.63 ± 6.77 vs. 16.45 ± 4.87 μmol/L), and nitrotyrosine-bound Cp (11.89 ± 9.29 vs. 5.85 ± 2.01 nmol/L) compared to controls. Low serum ferroxidase I activity was also present and appeared to be due to the oxidative changes in Cp and closely related to low antioxidative capacity. Those with advanced HF had the lowest ferroxidase I activity and the greatest mortality in two years. After adjustment, ferroxidase I activity emerged as a mortality predictor (HR: 2.95). Decreased Cp ferroxidase activity was a consequence of oxidative changes which could affect the progression of left ventricular dysfunction and mortality risk (63).

Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic relapsing inflammatory disease of the intestine with two major subtypes, Crohn's disease (CD) and ulcerative colitis (UC). It is considered a multifactorial disease with prominent inflammation involving extensive recruitment of blood neutrophils and, to a lesser extent, monocytes. Epithelial injury in IBD is characterized by the accumulation of activated neutrophils within epithelial crypts, their transepithelial migration, the release of large amounts of MPO and generation of reactive oxygen and nitrogen species (31, 64).

Elevated MPO with an imbalance between ROS production and antioxidative protection results in OxS which is considered an important pathogenic factor in IBD. The unregulated build-up of inflammatory ROS in the intestinal mucosa easily overwhelms antioxidant enzymes and establishes its persistence. Besides modulation of cellular signaling, ROS are epigenetic regulators that can change microRNAs expression (*e.g.* H_2O_2), which is considered one of the key elements in the pathogenesis of CD (31, 64, 65). The content of two antioxidant enzymes that carry Cu and Zn was found decreased in mucosa of IBD patients, with further deterioration following inflammation (66).

Significantly higher SCL was determined in patients with UC than in healthy controls, both in active disease and remission. Copper levels positively correlated with Cp and C3 and C4 complement (41). During the active inflammation, serum Cp and copper levels are expected to be higher in all IBD patients than in remission, after which they go back to normal. However, in some cases copper deficiency is determined. In a study that explored alteration of serum trace elements in IBD patients, copper insufficiency was present in 15.6% of all IBD cases (20.4% of CD and 7.1% of UC) most of them being in remission (65%). CRP values positively correlated with SCL and Cu/Zn ratio, while systemic inflammation increased the Cu/Zn ratio, suggesting a novel parameter for IBD (66).

There are a few described cases of association between Wilson's disease and UC, where both diseases display high SCL (41). Given the decreased content of antioxidant enzymes and possible alterations in copper metabolism, such as the decreased function of Cu-transporters, it is hypothesized that copper overload and extracellular accumulation take place in the intestinal mucosa, leading to the exacerbation of inflammation and excessive OxS. Extracellular Cu deposits were enriched in sulfur and iron, the latter being known for its ROS forming properties (18, 41).

Recent studies underscore the importance of the damaged epithelial barrier and dysregulated innate immune system in IBD pathogenesis. Macrophages have an important role in maintaining intestinal homeostasis by regulating immune responses to commensal flora. Experimental studies showed that macrophages play a protective role in the development of colitis. A protective anti-inflammatory role of macrophage-derived Cp was demonstrated in a mice model of IBD, but not liver-derived plasma Cp. The Cp-/- mice had exacerbated inflammatory response, increased mortality, and a higher degree of protein oxidation, which is likely due to uncontrolled activity of neutrophils MPO. The recruited macrophages were the primary source of colon Cp with an important role in maintaining intestinal homeostasis (15). Accordingly, several

therapeutic approaches have been investigated and gave beneficial results, such as overexpression of Cu/Zn-SOD in transgenic mice, inhibition of MPO, or inhibition of TNF-α (64, 67).

Interestingly, delayed apoptosis in neutrophils and other pro-inflammatory cells is present in IBD (64). We have already mentioned the role of Cp in the regulation of delayed neutrophil apoptosis. However, this process needs further investigations regarding IBD pathogenesis.

Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease with multifactorial etiopathogenesis. It is well documented that RA patients have increased serum copper and Cp levels compared to healthy controls, and those with active disease have significantly higher levels than in inactive RA. Copper and Cp might be implicated in the pathogenesis of RA (43, 68-71).

In a group of RA patients, treated with NSAIL, significantly higher values of copper and Cp were determined compared to healthy controls, specifically for total copper (153.9 ± 5.0 vs. 104.0 ± 1.8 μg/dl), immunoreactive Cp (39.0 ± 1.5 vs. 31.3 ± 0.6 mg/dl), oxidase activity of Cp (192.6 ± 6.9 vs. 125.7 ± 2.3 U/L), and the copper/immunoreactive Cp relationship (3.8 ± 0.04 vs. 3.3 ± 0.03 μg/mg). There was a significant negative correlation between Cp concentration and its activity compared to serum OxS markers (TBARS), while without differences in non-Cp bound copper (68).

Copper and Cp positively correlated with CRP values, while copper also correlated with erythrocyte sedimentation rate. Increased SCL was accompanied by the decreased zinc and HDL-c levels in these patients, as well as resulting rise in urinary copper excretion (hypercupreuria) in urine (8.00 vs. 3.98 mg/dl). It should be mentioned, that there was no influence of certain RA treatments on SCL, such as methotrexate, NSAIL, glucocorticoids, vitamin D3, or sulphasalazine (43, 69, 70).

In another study, SCL was significantly higher in patients with RA than healthy controls (110.2 ± 27.8 vs. 81.1 ± 32.1 μg/dl) and patients with osteoarthritis (95.4±27.4 μg/dl). The study determined higher copper levels in the synovial fluid of RA patients compared to healthy (56.6 ± 34.8 vs. 28.1 ± 7.1 μg/dl), which correlated to the lower selenium concentrations (72). Also, levels of Cp, MPO, and thrombin were markedly increased in the synovial fluid of RA patients. Moreover, Cp becomes proteolytically degraded during the inflammation. Also, thrombin cleaves Cp at a site of its inhibitory function toward MPO, thereby interfering with its antioxidant activity (36).

Some studies evaluated and found significant alterations in copper content in the hair of RA patients compared to healthy controls, but their results were contradictory (43, 69). Significantly lower copper levels were determined in erythrocytes and peripheral blood mononuclear leucocytes (PMBL) of RA patients compared to controls. Low erythrocyte Cu content is presumed to be due to the lower activity of SOD enzyme, perhaps as a consequence of its increased utilization in coping with the inflammation-induced OxS. Copper in PBMC was markedly lower in RA patients than in controls (74.3 ± 38.2 vs. 104.2 ± 8.5, μg/10^6 cells) and in those with active compared to inactive disease (58.0 ± 43.2 vs. 86.4 ± 33.2), with inverse relation of SLC and PBMC copper levels (69, 70).

Alterations in copper metabolism in RA seem to be caused by the change in its distribution, due to the accumulation in the inflamed areas and decreased bioavailability in other tissues. Overall, it seems that despite hypercupremia there is a copper deficiency in RA patients. Which, according to some authors may predispose to the higher incidence of infections in these patients (43).

Copper and Cp in leukemia and lymphomas

Numerous studies have found dyshomeostasis of trace elements during carcinogenesis. Owing to its effects on many cellular mechanisms, copper metabolism is often altered in tumors. Here, we briefly review current evidence of copper and Cp relevance in neoplasms of the immune system. In general, patients with malignancies have a significant increase in the total SCL and Cp activity compared to healthy individuals. There are some implications that copper might be included into the pathogenesis of certain forms of cancers due to its necessity for cellular growth (44, 73-76).

Copper and zinc are essential for normal lymphocyte proliferation, maturation and immune functions. Effects of copper and Cp on immunopathogenesis of neoplasia encompass a wide range of potential roles that can be viewed as a part of the antioxidant protection, iron homeostasis, regulation of innate immune response, regulation of angiogenesis and reparation (10, 33). Cellular iron depletion is of particular relevance and an effective host defense mechanism against neoplasia, same as the regulation of redox balance (10, 23). As mentioned, local changes in copper concentration influence the activity of NF-κB and HIF-1α, and thus indirectly on the expression of dozen of genes important for the immune response. Overexpression of HIF-1α and resulting angiogenesis are associated with cancerogenesis, while copper deprivation slows down this process (4, 50). Tumor-associated M2 macrophages (TAM) produce growth factors and have greater release of stored iron, by which they support tumorogenesis. In this regard, the VEGF-C – SOD3 axis was identified as a crucial mediator of tumor survival and metastasis. Although SOD3 levels are usually decreased in tumors, an inverse relationship is found in aggressive tumors, where malignant cells actually create and use SOD3 for survival (49, 52).

Significantly increased SCL is frequently determined in patients with acute and chronic leukemia compared to healthy individuals (73). In a study with a Greek cohort of patients with leukemia, increased copper concentration was the most abundant in cases. Also, the levels were markedly higher in acute leukemia (AML and ALL) compared to chronic (CML, CLL, and lymphoma) (74). In addition, elevated SCL was associated with disease relapse or progression, whereas normal SCL were

found in remission (18 vs. 10 mg/dL) (75). These findings indicate that copper alterations are dependent on tumor activity and may change in response to therapy.

Recent studies performed a high-precision isotopic analysis for determining alterations in trace elements. Blood levels of a heavier copper isotope 65Cu tend to decrease in patients with hematologic malignancies along with the serum enrichment with the light 63Cu isotope (76). In a study of untreated AML patients, although SCL were higher than in controls they were not significantly different (19.5 vs. 16.4 μmol/L). However, the isotopic abundance ratio of copper, 65Cu/63Cu, showed significantly lower levels in patients vs. controls (-0.35 vs. -0.11 per-mille). This is proposed to result from the extensive oxidative chelation of copper heavy isotope by cytosolic lactate in cancer cells (77, 78). Increased intratumoral copper concentrations might promote tumor growth or confer resistance to treatment. For example, the acquisition of resistance to platinum-based treatment was associated with the changes in copper efflux. The copper isotopic ratio is proposed for an early diagnostic use as a cancer biomarker and a follow-up marker (7).

Similar observations were reported in lymphoma patients. Serum copper levels had a very high specificity (95.3%), positive predictive value (92.9%), and a good sensitivity (61.9%) in the diagnosis of Hodgkin lymphoma (HL), with the cut off value at 25.04 μmol/L. However, there was no correlation with the type of HL nor a degree of spreading, assessed through the clinical stage (9). Earlier studies already have shown that the SCL is markedly higher in patients with malignant lymphoma before treatment compared to patients in complete remission (22.97 ± 1.55 vs. 16.36 ± 1.06 μmol/L). And with no marked difference between those in complete remission compared to controls (15.67 ± 0.98 μmol/L) (79).

Importantly, mean copper levels were not significantly different between non-Hodgkin and HL patients. Besides copper (108.5 μg/dL), nickel, chromium and cadmium were found in significantly higher contents in the blood and scalp hair of the lymphoma patients than in healthy controls. In addition, SCL was higher in nodular sclerosing lymphoma patients, in the hair in diffuse large B-cell lymphoma patients, and average copper levels were markedly higher at stage I (44).

Other disorders with copper and Cp alterations

Many other diseases are associated with altered SCL and/or Cp activity, however detailed discussion about all processes is beyond the scope of this article. The copper-based oxidase function of Cp is essential for neuronal development and antioxidant protection in the brain. Also, membrane bound Cp is expressed and secreted by astrocytes and is involved in inflammatory reactions (10, 15, 80). Considering these effects, alterations in copper regulation facilitate the iron accumulation and OxS, leading to neuroinflammation and neurodegeneration. Elevated SLC and/or Cp are frequently reported in Alzheimer's and Parkinson's diseases (15, 20, 25). Hypercupremia is determined in primary biliary cholangitis and sclerosing cholangitis, where copper accumulation may result in toxicity to the basal ganglia (81).

Estrogen and progesterone have well established enhancing effects on Cp synthesis in the liver. Therefore, the levels of Cp rise during pregnancy (almost double), and in females who take oral contraceptives (10, 22, 32). In placenta, there are soluble, GPI-anchored, and specific 4 amino acids longer Cp forms, the later selectively expressed in syncytiotrophoblasts (10). Roles of Cp in implantation and placental function are supposed to enhance iron transport, regulate vascular tone, and have immunomodulatory functions. Ceruloplasmin is determined to decrease the intensity of respiratory burst of neutrophils in the uterus of pregnant females (82). In pre-eclampsia, serum Cp levels are higher than in healthy controls (62 vs. 47 mg/dL), as well as Cp specific antioxidant activity. This increased Cp expression seems to be triggered by the local anoxic response (83).

Conclusion

Copper and Cp involvement in regulation of immune processes are directed toward regulation of iron metabolism, anti- or pro-oxidant activities, microbicidal and protective functions, apoptosis, tissue repair, etc. An increase in total SCL and Cp levels are frequent in the acute phase response to infection and inflammation. Also, they may reflect and take part in the pathophysiological mechanisms involved in DM and MI. Copper dyshomeostasis has been clearly established in many inflammatory autoimmune diseases, cancers, neurological and obstetric disorders. Besides disease understanding, irregularities in SCL could in some situations be utilized as a prognostic and predictive markers, or might be exploited as a therapeutic strategy in the future.

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Pregledni rad

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PLAVI "MOONLIGHTING" PROTEIN U IMUNSKOM ODGOVORU: ULOGE BAKRA I CERULOPLAZMINA U PATOGENEZI ZAPALJENJA I IMUNSKI POSREDOVANIH BOLESTI

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Povećanje nivoa serumskog bakra i/ili njegovog glavnog prenosioca u krvi – ceruloplazmina (Cp) čest je nalaz u nekim bolestima kod ljudi. Jedna od najpoznatijih uloga Cp je regulacija ćelijskog unosa gvožđa u situacijama hipoksije. Međutim, pored toga, bakar i Cp uključeni su u brojne fiziološke procese, kao što su redoks balans, regulacija transkripcijskih faktora, rast neurona, određene imunske funkcije: mikrobicidna aktivnost, citoprotektivna barijera, proliferacija limfocita i drugo. Ceruloplazmin je reaktant akutne faze zapaljenja, usled čega njegova koncentracija raste u situacijama akutnih infekcija ili zapaljenja. Takođe, narušavanje homeostaze bakra jasno je ustanovljeno u mnogim zapaljenskim autoimunskom bolestima, malignitetima, neurološkim i opstetričkim bolestima. Promene u metabolizmu bakra i Cp prisutne su u patogenezi dijabetesa melitusa i kardiovaskularnih bolesti. Pored toga, promene serumskog bakra mogu se iskoristiti kao prognostički i prediktivni biomarkeri. Međutim, interpretacija ovih podataka nije dovoljno u upotrebi u rutinskoj kliničkoj praksi. Iz tog razloga, cilj našeg rada bio je prikazati trenutna saznanja i najnovije dokaze o ulogama bakra i Cp, kao dela imunskog odgovora u etiopatogenezi brojnih bolesti, kao i prikazati koristi interpretacije njihovih promenjenih vrednosti.

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Ključne reči: *zapaljenje, makrofagi, limfociti, homeostaza gvožđa, oligoelementi*