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ROLE OF REDOX METALS, OXIDATIVE PROTEIN PRODUCTS AND ANTIOXIDANT POTENTIALS OF THIOLS IN DIABETIC RETINOPATHY

ULOGA REDOKS METALA, PROIZVODA OKSIDACIJE PROTEINA I ANTIOKSIDANTNIH POTENCIJALA TIOLA U DIJABETESNOJ RETINOPATIJI

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Summary: Oxidative stress has been proved in the pathogenesis of diabetes mellitus (DM) and diabetic retinopathy (DR) not only by the reactive oxygen species (ROS) but also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired glutathione metabolism, alteration in the antioxidants and advanced oxidative protein product formation. The current study was undertaken to establish the relationship between iron, copper and antioxidants like reduced glutathione (GSH), total thiols, and advanced oxidation protein products (AOPP) as well as total protein and albumin. The study group consisted of a total of 90 subjects which included non-diabetic healthy controls (n=30), diabetes mellitus patients (n=30), and diabetic retinopathy patients (n=30). All the parameters were measured using spectrophotometric methods. AOPP levels showed a very highly significant increase in DR patients and in DM patients compared to normal controls, the AOPP levels being higher in the DR compared to the DM patients (p= 0.001). The levels of thiols showed a very highly significant decrease in DR and DM as compared to normal subjects. The total proteins level showed a very highly significant decrease (P = 0.001) in DR and DM compared to normal. There was no change in the level of albumin. A significant increase in the levels of iron was observed in DR when compared to DM and control. The levels of copper in DR showed a very highly significant increase when compared to DM and controls (p = 0.001). Our study indicates a possible increase in the copper and iron-mediated generation of ROS thereby leading to increased consumption of antioxidants in the body.

Keywords: diabetic retinopathy, oxidative stress, protein oxidation, thiols

Kratak sadržaj: Prisustvo oksidativnog stresa u patogenezi dijabetesa (DM) i dijabetesne retinopatije (DR) dokazano je ne samo zahvaljujući prisustvu reaktivnih vrsta kiseonika (ROS) već i zbog neenzimske glikozilacije proteina, autooksidacije glukoze, poremećenog metabolizma glutationa, promena u stvaranju antioksidanata i naprednih proizvoda oksidacije proteina. Ova studija sprovedena je kako bi se ustanovio odnos između gvožđa, bakra i antioksidanata kao što je redukovani glutation (GSH), ukupnih tiola i naprednih proizvoda oksidacije proteina (AOPP) kao i ukupnih proteina i albumina. Ispitivanu grupu činilo je ukupno 90 subjekata, odnosno zdravih kontrola (n=30), pacijenata sa dijabetesom (n=30) i pacijenata sa dijabetesnom retinopatijom (n=30). Svi parametri izmereni su pomoću spektrofotometrijskih metoda. Uočen je veoma značajan porast nivoa AOPP kod pacijenata sa DR i DM u odnosu na zdrave kontrolne subjekte, s tim što su nivoi AOPP bili viši u DR nego u DM (p=0,001). U poređenju sa zdravim ispitanicima, nivoi tiola bili su veoma značajno sniženi u DR i DM. Nivo ukupnih proteina bio je veoma značajno snižen (p=0,001) u DR i DM u poređenju sa kontrolnom grupom, dok u nivou albumina nije bilo promena. Značajan porast nivoa gvožđa uočen je u DR u poređenju sa DM i kontrolnom grupom. Nivoi bakra u DR pokazali su veoma značajan porast u poređenju sa DM i kontrolnom grupom (p=0,001). Naša studija ukazuje na potencijalni porast produkcije ROS uz posredstvo bakra i gvožđa usled koje dolazi do povišene potrošnje antioksidanata u telu.

Ključne reči: dijabetesna retinopatija, oksidativni stres, oksidacija proteina, tioli

Introduction

Diabetes mellitus is characterized by absolute or relative deficiencies in insulin secretion and/or insulin action associated with chronic hyperglycemia and disturbances of carbohydrate, lipid and protein metabolism. Long-term complications represent a major cause of morbidity and mortality in patients with diabetes mellitus (1).

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ROS produced during normal oxidative metabolism are eliminated by an efficient scavenging system, but an imbalance between the production and scavenging of ROS can result in excessive levels of molecular oxygen or ROS, causing increased oxidative stress (2).

Various studies done by Moussa (3) and Pi J. et al. (4) have shown that oxidative stress induced by ROS generated due to hyperglycemia plays a significant role in causing secondary complications in diabetes mellitus such as diabetic retinopathy. The retina, with a high content of polyunsaturated fatty acids, the highest oxygen uptake and glucose oxidation relative to any other tissue, is more susceptible to oxidative stress (5, 6). Previous studies have proved the role of transition metals like iron (7) and copper (8) in causing oxidative stress. Copper and iron are toxic in their unbound form and cause redox imbalance due to their highly redox-active nature, which in turn leads to the activation of stress sensitive intracellular signaling pathways through the Haber-Weiss and Fenton reaction (9, 10). Major antioxidant activity of the body is contributed by the total thiols and the major contribution of total thiol pool is from the GSH, and free thiol groups present on protein albumin (11). AOPP is used as a sensitive assay for the oxidative damage to proteins (12).

Thus, the current study was designed to establish the relationship of trace metal ions like iron and copper with the antioxidants GSH, total thiols and AOPP along with the total proteins and albumin levels.

Materials and Methods

The study group consisted of a total of 90 subjects which included 30 healthy controls, 30 type 2 diabetic patients (13 males and 17 females) without any complications and 30 diabetic retinopathy patients (14 males and 16 females) in the age group between 40–60 yrs of both sexes. The thirty age and sex matched healthy individuals chosen as controls had no history of diabetes mellitus, hypertension, epilepsy, acute or chronic inflammatory conditions, psychiatric disorders, history of drug intake, smoking and alcohol consumption.

Type 2 diabetic patients were diagnosed based on the history, biochemical investigation and according to the biochemical criteria laid down by the WHO (13). Diabetic patients recruited for the study had no secondary complications of diabetes and they were all on oral hypoglycemic drugs. Diabetic retinopathy patients had no other secondary complications of diabetes. None of the subjects were on antioxidant supplements. History of all the subjects regarding the duration of diabetes, presence or absence of hypertension or dyslipidemia was taken into account.

Cases of diabetic retinopathy were diagnosed by the ophthalmologist with ophthalmoscopy. The cases included both proliferative and non-proliferative diabetic retinopathy. The study protocol was approved by the institutional ethical committee and informed consent was obtained from all subjects. Five mL of venous blood was collected in a heparinized vacutainer under aseptic precautions. The blood samples were centrifuged at 3000 rpm for 10 min. Plasma was separated for the assessment of advanced oxidative protein products (AOPP) by a modified Witko's method (14), where 2 mL of suitably diluted plasma was made to react with 200 μL of 1.16 mol/L and 0.04 mL of acetic acid. OD was measured immediately at 340 nm against blank. Total protein and albumin were determined by the Biuret method (15), total thiols by GL Ellman's procedure (16), reduced glutathione by the Ernest Beutler method (17), plasma iron by the dipyridil method of Ramsay (18), and plasma copper by the spectrophotometric method (19).

Statistical analysis was done using the Mann-Whitney U test.

Results and Discussion

The obtained results for markers of oxidative damage and redox metals are shown in *Table I*.

Table I Markers of oxidative damage and redox metals (MEAN±SD)

	Control (n = 30)	DM (n = 30)	DR (n = 30)
Total thiols (mmols/L)	0.448±0.06	0.333±0.03[a]	0.248±0.05[b, c]
GSH (mmols/L)	0.275±0.02	0.118±0.03[a]	0.116±0.02[b]
Total proteins (g/L)	76.0±7.0	66.6±0.02[a]	65.8±9.5[b]
Albumin (g/L)	38.4±5.7	38.2±7.7	39.2±5.8
AOPP (mmols/L)	0.017±0.00	0.275±0.07[a]	0.352±0.06[b, c]
Iron (μmol/L)	21.53±5.66	24.71±5.02	28.61±5.87[b, d]
Copper (μmol/L)	8.04±3.04	12.0±2.84[a]	12.43±2.8[b]

DM = diabetes mellitus, DR = diabetic retinopathy
a : p = 0.001 Control vs. DR, b: p = 0.001 Control vs. diabetes mellitus (DM)
c: p = 0.001 DM vs. DR, d: p = 0.01 DM vs. DR

There was a significant increase in copper ($p=0.001$) and the AOPP ($p=0.001$), and decrease in the GSH ($p=0.001$), total thiols ($p=0.001$) and total proteins ($p=0.001$) in the diabetic patients without complications when compared with normal controls. Further, an increase in copper ($p=0.001$), iron ($p=0.001$) and AOPP ($p=0.001$) and decrease in GSH ($p=0.001$), total thiols ($p=0.001$), total proteins ($p=0.001$) were found in diabetic retinopathy patients when compared to the controls. The present study showed a significant increase in AOPP and iron and a decrease in total thiol levels in the DR group in comparison with DM.

All forms of life maintain a reducing environment within their cells. The cellular redox environment is preserved by enzymes that maintain a reduced state through a constant input of metabolic energy. Disturbances in their normal redox state can cause a toxic effect through the production of peroxides and free radicals, that damages all the components of the cell including proteins, lipids and DNA. Since free radicals cause damage to lipids, protein and DNA, their oxidative product levels in the plasma or serum are increased in diseased conditions.

Diabetes is initially characterized by a loss of glucose homeostasis. The disease is progressive and is associated with a high risk of micro and macrovascular complications because of the abnormalities in the regulation of peroxide and transition metal metabolism (20). Studies by Yamagishi et al. (21) and Srivatsan et al. (22) point out the role of oxidative stress in the complications of diabetes like diabetic retinopathy.

Advanced oxidation protein products (AOPP) are a novel oxidative stress marker of protein along with oxidation of plasma thiol groups, termed »thiol stress«. It is quantitatively a major manifestation of protein oxidation due to oxidative and carbonyl stress on proteins and increase in global inflammatory activity (23) and may act as an inflammatory mediator. In our study we have observed a significant increase in the AOPP levels in diabetes mellitus and diabetic retinopathy, more so in diabetic retinopathy compared to healthy controls. The present study is in agreement with the studies done by Kowluru et al. (24).

Antioxidants protect cells from the damage caused by the unstable molecule. The body produces antioxidants to defend itself. Glutathione, a reductant which conjugates with drugs to make them more water soluble, is involved in the amino acid transport (meister's cycle), and serves as a cofactor for some enzymatic reactions and as an aid in the rearrangement of protein disulphide bonds. Depletion of NADPH can cause a decrease in the concentration of reduced glutathione. A decline in GSH, an important cellular antioxidant, can increase oxidative stress. The sulphydryl of the GSH can be used to reduce peroxides formed during oxygen transport and when plasma gets exposed to an increasing attack of ROS, which may be the cause for the decrease in the levels of GSH (25, 26).

The thiol group plays a prominent role in antioxidant reactions, in the reaction of catalysis, regulation of electron transport and preserving the correct structure of proteins. The levels and mutual relations between different redox forms of thiols in plasma are decisive for the plasma redox capacity which determines its proper functions. Plasma thiols include homocysteine, glutathione and cysteine. Due to the autooxidation or formation of mixed disulphides, their levels are decreased. In our study reduced glutathione and total thiols were significantly decreased in both diabetes mellitus and diabetic retinopathy when compared with normal subjects. This study goes in agreement with the studies done by Harnett et al. (27). A range of low molecular mass thiols have been shown to inhibit dicarbonyl adduction and cross-linking of the thiol-free protein lysozyme, consistent with these thiols being alternative (sacrificial) targets of glycation. Some of these thiols are more efficient modulators of glycation than the established glycation inhibitors such as amino-guanidine (28). A significant decrease in the levels of total proteins can be attributed to the increased formation of AOPP and decreased levels of GSH and total thiols.

Iron plays a central role in many metabolic processes. The most important property of free iron is its capacity to be reversibly oxidized and reduced, at the same time making it a highly prooxidant molecule. In this regard iron is able to generate powerful ROS. For this reason careful control of iron availability is crucial to the maintenance of normal cell function in the retina. In the diabetic eye there is an impairment of iron homeostasis leading to iron overload (29). The mechanisms involved are the destruction of heme molecules induced by hyperglycemia, intraretinal and vitreous hemorrhage, and overexpression of the renin–angiotensin system could contribute to iron overload in DR (30–32).

Insulin influences the iron uptake, utilization and storage by increasing the cell surface transferring receptors. Iron causes hyperinsulinemia by decreasing the insulin uptake and metabolism by hepatocytes. Further, the metal ion catalyzed protein oxidation is the basis of biological mechanisms for regulating changes in enzyme levels in response to shifts from anaerobic to aerobic metabolism and probably from one nutritional state to another. Free iron in its ferric state can be reduced to the ferrous state by various agents like superoxide anion, ascorbate and by glucose induced reduction by NADPH dependent reactions.

Studies done by Gao et al. (33) and Yamagishi et al. (34) have shown abnormal metabolism of zinc, iron and copper in diabetes and diabetic retinopathy.

A significant increase in free iron in the ferric state with a decrease in thiol has been shown in diabetic cases under poor glycemic control (35). It appears that increased oxidative stress in the presence of hyperglycemia may lead to the increased availability of transition metals like copper released from its storage site. Copper in its free form is a potent cytotoxic element, and because of its redox chemistry it readily participates in the Fenton and Haber-Weiss reactions to generate ROS (36–39).

The consequences of iron overload in the diabetic eye are complicated to evaluate because of the multiple forms of iron with different reactivity and several proteins that modulate their levels and actions. However, among the potential mechanisms of iron induced damage, it seems that oxidative damage is the most important. In addition, it should be noted that the iron ion catalyzes the binding of advanced glycation end products to a specific receptor, which is the crucial step in the pathogenesis of DR. Cell culture studies revealed that free iron stimulates the expression of adhesion molecules and monocyte endothelial adhesion (40), the key steps in the development of DR. It has been demonstrated *in vitro* that hyperglycemia causes complete destruction of the heme molecules from Hb and myoglobin, releasing free iron into the interstitial space. The present study shows an increase in the level of iron in circulation and also oxidative damage to the protein, where an increase in the levels of AOPP is observed parallel to the iron levels. Previous findings suggest that DM is associated with decreased ceruloplasmin and copper levels in serum (8) and is characterized by a mutation in the ceruloplasmin (CPL) gene, resulting in an elevation of copper ions in the blood. The inhibition of CPL activity may be attributed to the fact that hyperglycemia may induce the release of Cu ions from Cu-containing enzymes such as SOD and CPL, resulting in the elevation of copper ions in the blood. When proteins are exposed to metal catalyzed oxidation (MCO) systems, few amino acids are modified. The available evidence indicates that the MCO systems catalyze the reduction of iron and O_2 to H_2O_2. These products react at the metal binding sites on the protein to produce ROS, which attack the side chains of amino acid residues at the metal binding sites (34).

Evidence is provided for the generation of a wide variety of protein-derived free radicals via metal activated reactions and decreased free radical scavenging actions, which may play an important role in the pathogenesis through radical-mediated reactions. Thus, studies aimed at exploring preventive strategies to control iron availability by enhancing the antioxidants, especially thiols, are necessary, since these control various cellular signaling proteins through the oxidation/reduction of specific sensor molecules.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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